The acquisition of anxiety and the impact of transcutaneous vagus nerve stimulation on extinction learning in virtual contexts

Angstakquisition und der Einfluss transkutaner Vagusnervstimulation auf Extinktionslernen in virtuellen Kontexten

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Abbreviations

5-HT	5-Hydroxytryptamin or serotonin
ABVN	auricular branch of the vagus nerve
ACC	anterior cingulate cortex
ACh	acetylcholine
AD	Alzheimer's disease
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
BAT	behavioral avoidance test
BDNF	brain derived neurotrophic factor
BLA	basolateral amygdala
BNST	bed nucleus of stria terminalis
BOLD	blood oxygenation level dependent
CeA	central amygdala
CRF	corticotropin-releasing factor
CRN	cochlear root neurons
CS	conditioned stimulus
CS-	safety cue
CS+	fear cue
CTX-	safety context
CTX+	anxiety context
CVN	cervical vagus nerve
DA	dopamine
DSM	Diagnostic and Statistical Manual of Mental Disorders
ECG	electrocardiogram
EDA	electro-dermal activity
EMG	electromyography
EPN	early posterior negativity
ERP	event-related potential
FDA	Federal Drug Association
fMRI	functional magnetic resonance imaging

GABA	gamma-aminobutyric acid
GAD	generalized anxiety disorder
GCTX	generalization context
HMD	head-mounted display
HPA	hypothalamus-pituitary-adrenal
HR	heart rate
HRV	heart rate variability
IAPS	international affective picture system
IASP	International Association for the Study of Pain
IL	infralimbic cortex
ISI	Inter-Stimulus-Interval
ITI	inter-trial interval
LC	locus coeruleus
LPP	late positive potential
LTP	long-term potentiation
MDD	major depressive disorder
MEG	magnetoencephalography
mPFC	medial prefrontal cortex
NAcc	nucleus accumbens
NE	norepinephrine
NET	norepinephrine transporter
NMDA	N-methyl-D-aspartate
NS	neutral stimulus
NTS	nucleus of the solitary tract
OCD	obsessive-compulsive disorder
PAG	periaqueductal gray
PD	panic disorder
PnC	caudal pontine reticular nucleus
PTSD	post-traumatic stress disorder
QST	quantitative sensory testing

S1	primary somatosensory cortex
S2	secondary somatosensory cortex
sAA	salivary alpha amylase
SAD	social anxiety disorder
SCL	skin conductance level
SCR	skin conductance response
SEP	somatosensory evoked potentials
SSDR	species-specific defense reaction
ssVEP	steady-state visually evoked potential
STAI	State-Trait Anxiety Inventory
tDCS	transcranial direct current stimulation
TMS	transcranial magnetic stimulation
tVNS	transcutaneous vagus nerve stimulation
UR	unconditioned response
US	unconditioned stimulus
vmPFC	ventro-medial prefrontal cortex
VNS	vagus nerve stimulation
VR	virtual reality
VRET	virtual reality exposure therapy

Abstract

This thesis aims for a better understanding of the mechanisms underlying anxiety as well as trauma- and stressor-related disorders and the development of new therapeutic approaches. I was first interested in the associative learning mechanisms involved in the etiology of anxiety disorders. Second, I explored the therapeutic effects of transcutaneous vagus nerve stimulation (tVNS) as a promising new method to accelerate and stabilize extinction learning in humans.

For these purposes, I applied differential anxiety conditioning protocols realized by the implementation of virtual reality (VR). Here, a formerly neutral virtual context (anxiety context, CTX+) is presented whereby the participants unpredictably receive mildly aversive electric stimuli (unconditioned stimulus, US). Another virtual context (safety context, CTX-) is never associated with the US. Moreover, extinction of conditioned anxiety can be modeled by presenting the same contexts without US delivery. When unannounced USs were administered after extinction, i.e. reinstatement, the strength of the "returned" conditioned anxiety can provide information on the stability of the extinction memory.

In Study 1, I disentangled the role of elemental and conjunctive context representations in the acquisition of conditioned anxiety. Sequential screenshots of two virtual offices were presented like a flip-book so that I elicited the impression of walking through the contexts. Some pictures of CTX+ were paired with an US (threat elements), but not some other screenshots of the same context (non-threat elements), nor the screenshots depicting CTX- (safety elements). Higher contingency ratings for threat compared to non-threat elements revealed elemental representation. Electro-cortical responses showed

larger P100 and early posterior negativity amplitudes elicited by screenshots depicting CTX+ compared to CTX- and suggested conjunctive representation. These results support the dual context representation in anxiety acquisition in healthy individuals.

Study 2 addressed the effects of tVNS on the stabilization of extinction learning by using a context conditioning paradigm. Potentiated startle responses as well as higher aversive ratings in CTX+ compared to CTX- indicate successful anxiety conditioning. Complete extinction was found in startle responses and valence ratings as no differentiation between CTX+ and CTX- suggested. TVNS did not affect extinction or reinstatement of anxiety which may be related to the inappropriate transferability of successful stimulation parameters from epilepsy patients to healthy participants during anxiety extinction.

Therefore, in Study 3 I wanted to replicate the modulatory effects of tVNS on heart rate and pain perception by the previously used parameters. However, no effects of tVNS were observed on subjective pain ratings, on pain tolerance, or on heart rate. This led to the conclusion that the modification of stimulation parameters is necessary for a successful acceleration of anxiety extinction in humans.

In Study 4, I prolonged the tVNS and, considering previous tVNS studies, I applied a cue conditioning paradigm in VR. Therefore, during acquisition a cue (CS+) presented in CTX+ predicted the US, but not another cue (CS-). Both cues were presented in a second context (CTX-) and never paired with the US. Afterward, participants received either tVNS or sham stimulation and underwent extinction learning. I found context-dependent cue conditioning only in valence ratings, which was indicated by lower valence for CS+ compared to CS- in CTX+, but no differential ratings in CTX-. Successful extinction was indicated by equal responses to CS+ and CS-. Interestingly, I found reinstatement of conditioned fear in a context-dependent manner, meaning startle response was

potentiated for CS+ compared to CS- only in the anxiety context. Importantly, even the prolonged tVNS had no effect, neither on extinction nor on reinstatement of context-dependent cue conditioning. However, I found first evidence for accelerated physiological contextual extinction due to less differentiation between startles in CTX+ compared to CTX- in the tVNS than in the sham stimulated group.

In sum, this thesis first confirms the dual representation of a context in an elemental and a conjunctive manner. Second, though anxiety conditioning and context-dependent cue conditioning paradigms worked well, the translation of tVNS accelerated extinction from rats to humans needs to be further developed, especially the stimulation parameters. Nevertheless, tVNS remains a very promising approach of memory enhancement, which can be particularly auspicious in clinical settings.

Zusammenfassung

Ziel dieser Arbeit war es, die zu Grunde liegenden Mechanismen von Angst- sowie Trauma- und belastungsbezogene Störungen besser verstehen zu lernen und neue Therapieansätze zu entwickeln. Dabei lag mein Interesse zunächst bei den assoziativen Lernmechanismen, die bei der Entstehung von Angststörungen involviert sind. Darüber hinaus untersuchte ich die therapeutischen Effekte der transkutanen Vagusnervstimulation (tVNS) als neue und vielversprechende Methode, um das Extinktionslernen bei Menschen zu beschleunigen und zu stabilisieren.

Zu diesem Zweck verwendete ich differenzielle Angstkonditionierungsparadigmen in virtueller Realität (VR). Dabei wird den Probanden ein neutraler virtueller Kontext (CTX) gezeigt, in dem sie unvorhersehbare, leicht schmerzhafte elektrische Reize (unkonditionierter Stimulus, US) erhalten. Durch die erlernte Assoziation wird dieser Kontext zum (Angstkontext, CTX+). Ein zweiter virtueller Kontext, in dem die Probanden nie einen US erhalten, wird deshalb zum Sicherheitskontext (CTX-). Die Extinktion konditionierter Angst wiederum kann im Labor nachgestellt werden, indem beide Kontexte ohne US dargeboten werden. Werden aber den Probanden nach der Extinktion unangekündigte US appliziert (Reinstatement), dann kann die Stärke der zurückgekehrten Angst Aufschluss über die Stabilität des Extinktionsgedächtnisses geben.

Mit diesem Modell untersuchte ich in Studie 1 die beiden Rollen der elementaren und der konjunktiven Repräsentation eines Kontexts während der Akquisition von konditionierter Angst. Nacheinander aufgenommene Bildschirmfotos zweier virtueller Büroräume wurden dabei wie in einem Daumenkino kurz hintereinander dargeboten, so

dass der Eindruck entstand durch die Räume zu laufen. Der US wurde gleichzeitig mit manchen Bildern des CTX+ präsentiert (Gefahrenelemente), jedoch nie mit andere Bilder des CTX+ (keine-Gefahrenelemente) und auch nie mit Bildern, die CTX- darstellten (Sicherheitselemente). Höhere Kontingenzratings für Gefahrenelemente im Vergleich zu keine-Gefahrenelemente sprachen für die elementare Kontextrepräsentation. Elektrokortikale Signale zeigten höhere Amplituden der P100 und der frühen posterioren Negativität, die von Bildschirmfotos des CTX+ im Vergleich zum CTX- evoziert wurden, und weisen auf konjunktive Kontextrepräsentation hin. Insgesamt unterstützen diese Befunde die duale Repräsentation eines Kontexts während der Angstakquisition bei gesunden Probanden.

Studie 2 thematisierte die Effekte der tVNS auf Extinktionslernen. Potenzierte Schreckreaktionen und aversivere Ratings in CTX+ verglichen mit CTX- sprachen für erfolgreiche Angstkonditionierung. Vollständige Extinktion wurde in der Schreckreaktion und in Valenzratings gefunden, da sich die Reaktionen auf CTX+ und CTX- am Ende dieser Phase nicht mehr unterschieden. Jedoch beeinflusste tVNS während der Extinktion weder das Extinktionslernen noch reduzierte sie die Wiederkehr der Angst. Aufgrund der Neuheit dieses Forschungsbereichs wurden Stimulationsparameter aus der Anwendung der tVNS bei Epilepsiepatienten gewählt. Die Übertragbarkeit auf gesunde Probanden während Angstextinktion blieb noch unklar.

Deshalb sollte in Studie 3 ein tVNS Effekt auf die Herzrate und die Schmerzwahrnehmung repliziert werden, und zwar mit genau diesen Stimulationsparametern. Die Ergebnisse zeigten jedoch, dass tVNS weder subjektive Schmerzratings, noch die Schmerztoleranz, noch die Herzrate der gesunden Probanden beeinflusste. Schlussfolgernd mussten in den folgenden Untersuchungen die tVNS

Parameter geändert werden, um eine erfolgreiche Wirkung der tVNS bei gesunden Probanden zu ermöglichen.

In Studie 4 verlängerte ich die Stimulationszeit und adaptierte das verwendete Konditionierungsmodell zu einem Furchtkonditionierungsparadigma in VR. Dabei wurde der US während der Akquisition durch einen Hinweisreiz (CS+) im Angstkontext angekündigt, nicht jedoch durch einen zweiten Hinweisreiz (CS-). Beide Hinweisreize wurden außerdem in einem zweiten Kontext (CTX-) dargeboten und nie mit einem US gepaart. Danach unterzogen sich die gesunden Probanden entweder einer verum tVNS oder einer Scheinstimulation und durchliefen Extinktionslernen. Kontext-abhängige Furchtkonditionierung fand ich nur in Valenzratings, da die Probanden CS+ im Angstkontext negativer bewerteten als CS- und gleich im Sicherheitskontext. Erfolgreiche Extinktion zeigte sich in gleichen Bewertungen des CS+ und CS-. Interessanterweise fand ich kontext-abhängige Angstwiederkehr, d.h. Schreckreaktionen waren nur in CTX+ für CS+ potenziert im Vergleich zum CS-. Die verlängerte Stimulationszeit der tVNS hatte keinen Effekt, weder auf die Extinktion, noch auf die Wiederkehr der kontext-abhängigen konditionierten Furcht. Außerdem zeigten sich erste Tendenzen zu beschleunigter Extinktion des Kontextlernens durch tVNS, da die Schreckreaktion zwischen CTX+ und CTX- in der tVNS Gruppe weniger differenziert ausfiel als in der scheinstimulierten Gruppe.

Zusammenfassend bestätigt diese Arbeit die duale Repräsentation eines Kontexts während der Angstakquisition auf neuronaler und subjektiver Ebene. Außerdem wurden erfolgreiche Angstkonditionierungs- und kontextabhängige Furchtkonditionierungs-paradigmen etabliert. Trotz keiner oder schwacher Effekte der tVNS auf Extinktion und Angstwiederkehr bleibt sie ein sehr vielversprechender Ansatz der Gedächtnissteigerung, der vor allem für den klinischen Kontext relevant ist.

1 Theoretical background

1.1 Recognizing threat – evolutionary mechanisms for survival

The appropriate response to various circumstances in the environment is an essential evolutionary mechanism for the survival of individuals and entire species. For fast and correct decisions, organisms have to make, a theory that combines two basic motivational systems has been developed: the appetitive and the defensive system (Bradley, Codispoti, Cuthbert & Lang, 2001; Davis & Whalen, 2000; Fanselow, 1994). The appetitive system is active for sustenance and recreation, it induces ingestion, caregiving, and copulation (Bradley et al., 2001). In contrast, the defense system takes over, when any kind of threat is expected from the environment. Depending on the threat, such a situation requires a proper response for survival which might be withdrawal, escape or attack (Bradley et al., 2001). Interestingly, vertebrates share these two emotionally motivational systems which elicit a certain behavior (Janak & Tye, 2015). In the view of evolution, fear and anxiety are emotions that assure the survival of many individuals.

About 50 years ago, Martin Seligman (1970) proposed the *preparedness theory*, which describes an organism's ability to easily associate events that he is prepared for. On the other hand, the organism has difficulties to associate unprepared or even contraprepared events. According to Mineka and Öhman (2002), events that are evolutionary relevant for survival are particularly likely to become signals for threat. In other words, humans develop fears and phobias more likely if the threatening object or situation has phylogenetically been relevant for the survival of our ancestors, e.g. wide open spaces, predators, and heights (Mineka & Öhman, 2002). Such associative memory traces are difficult to extinguish, directed towards phylogenetically relevant distinct and non-

arbitrary stimuli, stable in its cognitive meaning (e.g. a non-venomous snake), and can be acquired in one concurrent appearance (Seligman, 1971). In sum, the motivational system and the preparedness of an organism support a fast decision of the appropriate response to threat, which is essential and indispensable for survival.

Fanselow (2018b) noted three critical factors, which boosted natural selection based on animal responses to threats. First, threat limits an animal's repertoire to a selection of responses, which in the view of phylogeny have been useful in previous similar situations. Second, fast associative learning mechanisms between a stimulus and a threatening event promote the recognition of threat and defensive response. Third, during threat learning, various dimensions of predator imminence are integrated into the most effective defense response.

Bolles (1970) supported the idea that a species possesses a certain genetically predetermined repertoire of behavior for responding to threat. Therefore, he described the species-specific defense reaction (SSDR). If the organism faces any threat which requires an immediate response, the innate behavioral repertoire for defense will be specifically retrieved which contains a selection of phylogenetically survival promising actions (Fanselow, 2018a). The behaviors underlie a hierarchical order, but it can change if a behavior has previously failed. An example is the defense behavior in rats, which includes freezing, fight or flight. Is a rat exposed to a predator, its behavioral repertoire is restricted to its SSDR. If the rat has previously fought against this predator and got very seriously hurt, this time flight might be the first behavior in hierarchy to avoid life-threatening consequences (Burghardt, Bush, McEwen & LeDoux, 2007; Deakin & Graeff, 1991; Lowry et al., 2008; McNaughton & Corr, 2004). Therefore, the rat integrates previous experiences in order to assure the most effective defense behavior for the specific situation.

The sequential defense reactions depend on the proximity or imminence of threat (Blanchard & Blanchard, 1989; Bradley et al., 2001; Fanselow, 1994; Masterson & Crawford, 1982; Mobbs et al., 2009; Timberlake, 1993). In his predator imminence model, Fanselow (1994, 2018a) describes the defensive behavior system on three different levels, which can activate different modes. Low levels of defensive responses are elicited by a distal threat like entering a potentially threatening area, which requires pre-encounter defensive mode. Behaviors like meal pattern reorganization or protective nest maintenance can be the consequence. Moderate levels of fear elicit post-encounter defense reactions due to a predator within sight. This fear is learned and, by activation of projections from the amygdala to the periaqueductal gray in rats, freezing behavior is activated (Fanselow, 1994). Extreme threat imminence demands active defenses. Such circa-strike defenses include physical contact with the predator, escape, threat displays or jump attacks. Midbrain structures like dorsolateral periaqueductal gray and superior colliculus play a crucial role here (Fanselow, 1994). However, such a life-threatening situation is never static. Hence, the rapid switch between post-encounter and circa-strike defensive modes, which are mediated via inhibitory interactions between the relevant brain structures mentioned above, is indispensable for the survival of such life-threatening situations (Fanselow, 1994; Perusini & Fanselow, 2015). Regarding this model, a distinction between fear and anxiety depending on the imminence of the threat has been established.

In sum, the defense system integrates previous experiences and limits the individual's behavioral repertoire to the most promising response for survival.

1.2 Fear and Anxiety

1.2.1 Neurobiology of fear and anxiety

In the course of evolution, one brain region developed mediating emotional processing, circuitry, and function: the amygdala (Janak & Tye, 2015; McDonald, 1998). Amygdala and close similar circuits were found even in non-mammalian species including reptiles, birds, and fish (Jarvis et al., 2005; Johnston, 1923; Lanuza, Belekhova, Martínez-Marcos, Font & Martínez-García, 1998). As a major player in the defensive system, the amygdala is described as the fear center of the brain (Davis & Whalen, 2000). Threat information is processed in the basolateral nuclei (BLA), which projects to the central nucleus of the amygdala (CeA) or to the Bed Nucleus of Stria Terminalis (BNST), respectively (Walker, Toufexis & Davis, 2003). CeA and BNST share similar cell morphology and are highly connected (Alheid et al., 1998). Though patients with amygdala lesion are still able to report the experience of fear after CO_2 inhalation (Feinstein et al., 2013) and after associative fear learning (Bechara et al., 1995), the amygdala plays a crucial role in the defense system in human species and in eliciting behavioral responses to threat. However, lesions of the amygdala lead to impaired fear recognition in patients (Adolphs et al., 2005). Therefore, the amygdala is the key brain structure, which is active during a response to threat (Davis & Whalen, 2000; Roozendaal, McEwen & Chattarji, 2009). Within this system, panic, fear, and anxiety are different constructs with distinct underlying mechanisms generating a distinguishable set of behaviors (Perusini & Fanselow, 2015). In fact, panic, fear, and anxiety can be classified on the continuum of the predator imminence model (Fanselow, 2018a; Lang, Davis & Ohman, 2000). First, panic is only elicited in an organism when a threat, e.g. a predator, is very close (Fanselow, 2018a; Perusini & Fanselow, 2015). Second, fear is defined as a phasic response to imminent threat evoked by a clearly identifiable threatening stimulus,

i.e. an explicit cue (Grillon, 2008). Here, the central amygdala is crucial for the response to discrete threatening stimuli, which elicit phasic fear (Alvarez, Biggs, Chen, Pine & Grillon, 2008; LeDoux, 2000). Regarding Fanselow's predator imminence model, fear would elicit post-encounter, but most importantly circa-strike defenses (Davis, Walker, Miles & Grillon, 2010; Fanselow, 1994). Hence, fear results in phasic body reactions including fight or flight, increased physiological arousal, alarm reactions or decreased threat impact by the reduction of pain sensitivity (Blanchard, Yudko, Rodgers & Blanchard, 1993; Bolles & Fanselow, 1980; Carlsson et al., 2006; Grillon, 2008; Rhudy & Meagher, 2000). Moreover, fear inhibits responses competing with threat reactions and narrowing attention to the threatening stimulus (Grillon, 2008; Mowrer & Aiken, 1954; Vuilleumier, 2005). Third, anxiety, also called sustained fear, is elicited by unpredictable threats and potentially dangerous contexts (Grillon, 2008). Anxiety is mediated by the extended amygdala, namely the BNST (Alvarez, Chen, Bodurka, Kaplan & Grillon, 2011; Davis & Shi, 1999; Davis et al., 2010; Sullivan et al., 2004; Walker et al., 2003). As the context is relevant for experiencing anxiety, the hippocampus is additionally suggested as crucial brain structure processing anxiety (Alvarez et al., 2008; Andreatta et al., 2015a; Hasler et al., 2007; Marschner, Kalisch, Vervliet, Vansteenwegen & Büchel, 2008). Considering pre-encounter and post-encounter defense behavior (Davis et al., 2010; Fanselow, 1994), anxiety is accompanied by an increase in overall sensory sensitivity (Baas, Nugent, Lissek, Pine & Grillon, 2004) and feelings of helplessness and insecurity (Grillon, 2002, 2008). Additionally, as the organism is not able to identify safe periods, the chronic stress level is increased (Seligman, 1968; Seligman & Binik, 1977).

In sum, these distinct responses to threat dependent on predator's imminence are crucial for survival and provide evidence for distinct and highly connected fear and

anxiety networks in the brain, which guarantee fast threat processing and the appropriate response.

1.2.2 Measures of fear and anxiety

Emotions like fear and anxiety elicit multiple responses in the human body (Dolan, 2002; Mauss, Levenson, McCarter, Wilhelm & Gross, 2005). Lang (1995) described three levels of affective responses: the behavioral, subjective and physiological level. Recently, two controversial theories on the neurobiological models of fear and threat are discussed. LeDoux and colleagues hold the opinion of the two-system framework of fear and anxiety (LeDoux & Pine, 2016). In this view, when an organism experiences any threat in the environment it recognizes the danger by a sensory system and responds on two levels: first, the cognitive circuit which mediates the fear response like subjective fearful feeling; second, the defensive survival circuit which refers to the bodily and behavioral changes according to the threat stimulus. They argue that fear or anxiety can only be measured on the cognitive level by asking a person about his or her fear. In contrast, the defense circuit regarding behavioral and physiological responses represents threat responses or defensive responses, but does not necessarily mean fear and anxiety. Their main argumentation lies in animal research since animals only respond on the defensive survival circuit when exposed to threat as they cannot express their momentary subjective feelings. On the other hand, Fanselow and Pennington (2018) state the opinion of a central fear generator/central neural circuit which is conserved in evolution across species and can best be described by the integration of responses on autonomic, behavioral and cognitive-behavioral levels to the dangerous stimulus or environment. Subsequently, physiological, behavioral and experiential components mediate emotions of fear and anxiety on all response levels (Fanselow, 2018a).

Regardless of the naming of threat response and fear or anxiety response of both theories, these affective responses can be measured on different levels in humans with the following methodologies: First, by confronting a person with a threatening stimulus behavioral patterns like fight or flight can be investigated. In the laboratory, behavioral avoidance tests (BAT) are frequently used (Meulders, Vansteenwegen & Vlaeyen, 2011; Shiban, Schelhorn, Pauli & Mühlberger, 2015) or decision-making tasks to approach or avoid threatening stimuli (Pittig, Schulz, Craske & Alpers, 2014). Moreover, fear- and anxiety-related questionnaires can describe the general behavior a person usually performs when he or she faces a threatening stimulus without the necessity to elicit emotion (Glotzbach-Schoon et al., 2013c; Schiele et al., 2016; Schienle, Scharmüller, Leutgeb, Schäfer & Stark, 2013). Second, the person's subjective judgment and evaluation of his or her emotional state can be investigated by self-reports. Such explicit ratings can, for instance, describe high scores of subjective states like arousal or fear in a threatening situation. Additionally, associative learning between a stimulus and threat can lead to declarative memory formation, which predicts the expectancy of the threat in the future. Hence, this threat expectancy or contingency can also be assessed by subjective ratings (Boddez et al., 2013). Third, affective responses on the physiological level are mediated by somatic and autonomic systems. This comprises psychophysiological responses including startle response, electro-dermal activity (EDA), and electrophysiological brain responses recorded by electroencephalography (EEG; Fowles, 1980).

Startle response. According to Koch *'startle is a fast response to sudden, intense stimuli and probably protects the organism from injury by a predator or by a blow'* (Koch, 1999, p. 107). Sudden acoustic, visual and tactile stimuli can elicit startle responses in many animals and humans (Landis & Hunt, 1939). Even fish startle due to olfactory stimuli (Pfeiffer, 1963). In fact, various species show startle responses which make startle a

powerful tool in translational studies (Fendt & Koch, 2013). A similar response pattern like a fast twitch of facial and body muscles (Koch, 1999) is generated in rats and humans by the same parameters of an acoustic startle stimulus (Koch, 1999). The magnitude and latency of the startle response are influenced by various parameters including stimulus intensity, inter-stimulus interval, ongoing motor behavior, genetic differences, diurnal rhythm, sensory environment and most importantly the emotional state (Koch, 1999). Vrana, Spence, and Lang (1988) investigated acoustic startle responses in humans while they looked at pleasant, neutral and unpleasant pictures. Indeed, startle magnitudes were highest when unpleasant pictures were shown and lowest when pleasant pictures were presented. Therefore, the authors concluded valence-dependent modulation of the startle magnitude independent of the arousal state (Vrana et al., 1988). Importantly, cognitive processes do not influence startle responses (Andreatta, Mühlberger, Yarali, Gerber & Pauli, 2010; Hamm & Weike, 2005), whereas darkness potentiates startle responses in humans (Grillon, 2002, 2008; Grillon, Pellowski, Merikangas & Davis, 1997; Mühlberger, Wieser & Pauli, 2008). Moreover, startle potentiation can be evoked by sensitization and most importantly fear conditioning, whereas startle attenuation can be induced by habituation, prepulse inhibition and positive emotional states (Davis, 1989; Guerra, Sánchez-Adam, Anllo-Vento, Ramírez & Vila, 2012; Koch, 1999). In regard to the predator imminence model, startle inhibition is expected for pre-encounter defenses when an organism experiences anxiety for letting the organism respond (Lang et al., 2000). Fear potentiation of the startle response only begins in the post-encounter phase and increases with approaching threat (Lang et al., 2000). The neural pathway of acoustic startle responses was mostly investigated in rats, mice, cats and is thought to be similar in humans (Koch, 1999). In a first step, an acoustic startle probe, i.e. a loud noise, is perceived via the ear. The signal is transferred to the cochlear nucleus and cochlear root neurons

(CRN) which project to the caudal pontine reticular nucleus (PnC). Importantly, the PnC is modulated by excitatory and inhibitory PAG neurons which perceive information from thalamus, hippocampus, BLA, and CeA. From PnC neurons project to the spinal cord or facial muscles to elicit the startle response (Shi & Davis, 2001; Simons-Weidenmaier, Weber, Plappert, Pilz & Schmid, 2006). In sum, the startle response is an amygdala-modulated valence-dependent response across species, which is potentiated in defensive behavior in response to imminent threat and attenuated in safe environments.

Electro-cortical signal. Early affect modulated stimulus processing can be investigated electro-cortically. Hans Berger's investigations of EEG in 1924 (see Jung & Berger, 1979) revolutionized research on early attention (Luck, Woodman & Vogel, 2000) and affective stimulus processing (for review see Olofsson, Nordin, Sequeira & Polich, 2008). Small voltage changes by the postsynaptic potentials in the cortical pyramidal cells can be recorded from the scalp (Miskovic & Keil, 2012; Woodman, 2010). Event-related potentials (ERPs) can be elicited by e.g. visual, auditory or somatosensory stimuli which trigger a distinct emerging pattern of voltage changes. The advantage of this method is the high temporal resolution (Luck et al., 2000), a disadvantage is the low spatial resolution (Luck, 1999).

Altogether, fear and anxiety can be monitored on different levels across species. Whereas animal research sticks to the behavioral and physiological measures of threat responses (LeDoux & Hofmann, 2018), human fear and anxiety measures include behavioral, subjective and physiological levels (Fanselow, 2018a; Lang, 1995). Investigations in humans can allow conclusions on behavioral level by behavioral tests, on the subjective level by asking the participants and on the physiological level by measuring changes in body and brain.

1.2.3 Pathological fear and anxiety

As reported above, fear and anxiety are inevitable for the survival of an individual. However, the maladaptation of fear and anxiety can become pathological. During their lifetime, up to 33.7% of the population are affected by an anxiety disorder (Bandelow & Michaelis, 2015). Wittchen et al. (2011) reported a 12 months prevalence of anxiety disorder in 14.0% of the population in 27 European countries plus Iceland, Norway, and Switzerland.

The term *anxiety disorder* comprises various diseases (American Psychiatric Association, 2013), some associated with fear others with anxiety and others share symptoms of both fear and anxiety (Grillon, 2008). More precisely, phasic fear is associated with specific phobias (Öhman & Mineka, 2001; Seligman, 1971), whereas sustained fear, i.e. anxiety, is associated with generalized anxiety disorders (GAD) and panic disorder (PD) with and without agoraphobia (Bandelow & Michaelis, 2015; Bouton, Mineka & Barlow, 2001; Grillon, 2008; Lonsdorf & Merz, 2017; Richter et al., 2012). Common behavior in anxiety patients is avoidance (Pittig, Treanor, LeBeau & Craske, 2018); patients report excessively high subjective fear and anxiety and they might also show exaggerated physiological responses to a perceived threatening stimulus or situation.

Notably, post-traumatic stress disorder (PTSD) was recently classified as trauma- and stress-related disorder (American Psychiatric Association, 2013) rather than an anxiety disorder. Within a year, 2.0% of the European population suffered from PTSD (Wittchen et al., 2011). PTSD patients share both symptoms of fear of a discrete stimulus, which they associate with the trauma, and symptoms of anxiety in terms of hypervigilance (Grillon, 2008). According to the diagnostic and statistical manual of mental disorders (DSM-5), symptoms for PTSD include re-experience of the trauma, particularly responding to

reminders of the trauma with emotional or physical distress, avoidance of any stimuli related to the trauma, exaggeration of arousal and startle responses and increase in negative affect (American Psychiatric Association, 2013). In order to diagnose PTSD, these symptoms must remain constant for at least one month and induce social and occupational problems (American Psychiatric Association, 2013).

Interestingly, among all people that have witnessed or experienced near-death, sexual violence, or a bad injury, only 10% will develop PTSD (Kessler, Sonnega, Bromet, Hughes & Nelson, 1995; Yehuda & LeDoux, 2007). For them, stimuli that are related to the traumatic experience like the context, sound, smell or sight can serve as intrusive and haunting reminders which might lead to nightmares or flashbacks in patients. A representative epidemiological study investigated the prevalence of PTSD in Germany and found a higher prevalence in persons older than 60 years than in younger ages, which the authors attribute to consequences of World War II (Maercker, Forstmeier, Wagner, Glaesmer & Brähler, 2008).

Several mechanisms are thought to be responsible for the development of anxiety and stress-related disorders. First, patients associate an aversive stimulus faster with a neutral stimulus than healthy controls. In other words, they learn threat associations easier than healthy persons (Orr & Roth, 2000). Second, abnormal safety learning in childhood (Britton, Lissek, Grillon, Norcross & Pine, 2011) and adulthood (Jovanovic, Kazama, Bachevalier & Davis, 2012; Lissek et al., 2005), as well as overgeneralization of fear responses (Dymond, Dunsmoor, Vervliet, Roche & Hermans, 2015; Lissek et al., 2014) can result in the development of an anxiety or stress-related disorder. Third, after the association of a stimulus with a threatening event, patients tend to avoid the stimulus, a behavior that prevents extinction learning (Mowrer, 1953). Additionally, individual differences including temperamental and biological factors can influence the

development of maladaptive anxiety (Lonsdorf & Merz, 2017; Mineka & Oehlberg, 2008). Individual genetic variability and therefore heritable genetic predisposition might explain that some patients develop an anxiety disorder and some do not (Hariri & Holmes, 2006; Mühlberger et al., 2014; Pezawas et al., 2005; Soliman et al., 2010).

From this clinical perspective, investigations on the underlying mechanisms of the development of fear and anxiety on the behavioral, subjective and physiological level are warranted for the understanding of the processes involved in the development of anxiety and trauma- and stress-related disorders, and further for the enlightenment of optimal individual treatment conditions.

1.3 Experimental models to investigate fear and anxiety

1.3.1 Fear conditioning in animals and humans

Associative learning mechanisms are widespread in animals. Especially the association of any kind of motivational stimulus with appetitive (e.g. food) or with aversive (e.g. threat) meaning is essential for survival (Bradley et al., 2001; Fanselow, 1994). In classical conditioning, a neutral stimulus (NS) is paired with an unconditioned stimulus (US), which elicits an unconditioned response (UR). After several repetitions of the pairing, the formerly NS becomes the conditioned stimulus (CS), as its presentation alone elicits the conditioned response (CR; Pavlov, 1927). In this manner, Ivan Pavlov conditioned a dog by pairing the ringing of a bell (CS) with food (US), which naturally elicited salivation (UR). After a few pairings, the presentation of the ringing bell alone elicited salivation (Pavlov, 1927). In classical conditioning, the modalities of conditioned and unconditioned stimuli as well as the organisms that are able to learn associations and can subsequently be conditioned, are diverse. In this way, larvae, as well as adult fruit flies (*Drosophila melanogaster*), learn to associate an odor with reward or punishment (Gerber & Hendel,

2006; Tanimoto, Heisenberg & Gerber, 2004). Honey bees (*Apis mellifera*) learn to show the proboscis extension reflex to the odor of flowers (Gerber et al., 1996) and mollusks like *Aplysia californica* learn the association of a tactile stimulus and an electric shock resulting in a withdrawal reflex due to tactile stimulation only after conditioning (Carew, Walters & Kandel, 1981). Strikingly for investigations of fear and anxiety, mammalian model organisms like rodents, including rabbits, mice, and rats, can be conditioned as well (Calhoon & Tye, 2015; Caro-Martín, Leal-Campanario, Sánchez-Campusano, Delgado-García & Gruart, 2015; Micale et al., 2017; Peña, Engineer & McIntyre, 2013; Steinmetz, Lavond & Thompson, 1989). Even humans showed appetitive and aversive conditioning to very basic geometric shapes which indicated associative learning and distinct reward and punishment networks in the brain (Andreatta et al., 2012; Andreatta & Pauli, 2015). In detail, three geometric shapes were presented on a computer screen. One was paired with a mildly painful electric shock, another was paired with chocolate or salty pretzels, and the third was presented without reinforcer (Andreatta et al., 2012). In line with the predictions, startle responses were attenuated for the positively connoted geometric shape and potentiated for the negatively connoted geometric shape (Andreatta et al., 2012). Notably, based on studies in rodents, classical fear conditioning is frequently used as a model to investigate fear in humans (Lonsdorf et al., 2017). The majority of animal studies use single cue conditioning protocols (Lonsdorf et al., 2017) in which only one stimulus is paired with an US (e.g. Peña et al., 2013). Single cue conditioning is barely used in humans (Wong & Lovibond, 2017). Human studies frequently apply differential fear conditioning paradigms (e.g. Andreatta et al., 2012). Here, one stimulus (CS+) is paired with the US, whereas another stimulus (CS-) of the same modality is never paired with the US. Differential cue conditioning protocols investigated the acquisition of fear in humans on various levels. Those studies defined risk alleles for anxiety disorders (Hettema, Annas,

Neale, Kendler & Fredrikson, 2003), elucidated differential psychophysiological, cortical, and subcortical responses to CS+ and CS- (Andreatta & Pauli, 2015; Fullana et al., 2015; Wieser, Flaisch & Pauli, 2014) and measured subjective fear responses as well as the degree of avoidance learning (Meulders et al., 2011). Therefore, cue conditioning serves as an established model for the simulation of acquisition of fear and can easily be implemented into the laboratory for investigations with animals, healthy humans, and patients with anxiety disorders, e.g. specific phobias.

From a neurobiological perspective, rat studies detected the amygdala as the key player in classical fear conditioning as the amygdala activity correlated with conditioning (Quirk, Armony & LeDoux, 1997). Studies in animals described the amygdala as a brain complex consisting of several nuclei including lateral, medial, cortical and central nuclei (Davis & Whalen, 2000; Maren, 2001). In detail, for fear conditioning, two distinct systems were described (LeDoux, 2012; Maren, 2001). First, the basolateral complex (BLA) consisting of lateral, basolateral and basomedial amygdala receives input from the respective sensory system i.e. from the auditory thalamus and auditory cortex for auditory CS, from the hippocampal formation for contextual CS, from perirhinal cortex for visual CS and from the vomeronasal organ for olfactory CS (LeDoux, 2012; LeDoux, 2000; Maren, 2001). Additionally, somatosensory thalamic and cortical pathways send input about the US (Maren, 2001). Therefore, the BLA integrates the CS-US information as well as forms and stores the CS-US associations during classical fear conditioning (Maren, 2001). Second, the central amygdala receives input from BLA and builds the interface to the appropriate and required fear response (Paré, Quirk & Ledoux, 2004). The central amygdala projects among others to BNST, hypothalamus, midbrain, medulla and initiates defensive behavior (Maren, 2001; Paré et al., 2004).

The investigation of underlying biological mechanisms of fear conditioning in humans is not possible on the same level as in animals. Despite these constraints, functional magnetic resonance imaging (fMRI) studies and brain lesion studies explored the human amygdala and found results consistent with the animal models (Phelps & LeDoux, 2005). During fear conditioning, fMRI studies showed blood oxygenation level-dependent (BOLD) response of the amygdala (Büchel, Morris, Dolan & Friston, 1998; LaBar, Gatenby, Gore, LeDoux & Phelps, 1998), whereby – in parallel with rats (Quirk et al., 1997) – the magnitude of the amygdala activity predicted the conditioned fear response (LaBar et al., 1998; Phelps, Delgado, Nearing & LeDoux, 2004). The investigation of the time course of amygdala activation during conditioning demonstrated higher amygdala activity to CS+ vs. CS- at the beginning of conditioning than at the end (Büchel et al., 1998; Straube, Weiss, Mentzel & Miltner, 2007). Moreover, patients suffering from amygdala lesions or damage showed impaired acquisition of conditioned SCR (Bechara et al., 1995), difficulties in the processing of fear-relevant stimuli like faces (Vuilleumier, Richardson, Armony, Driver & Dolan, 2004), impaired preferred processing of emotional stimuli like aversive compared to neutral words (Anderson & Phelps, 2001), and most importantly impaired acquisition of conditioned responses and impaired emotional memory network (LaBar, LeDoux, Spencer & Phelps, 1995). In sum, external but not internal (Feinstein et al., 2013) threat stimuli are processed in the amygdala which influences startle reflex and emotion modulation (Angrilli et al., 1996). Amongst other brain regions, the amygdala is activated in patients suffering from a specific phobia when a fear-relevant stimulus is presented, which shows the clinical relevance of this line of research (Straube, Mentzel & Miltner, 2005). Fear conditioning is an established and successful model for the understanding of fear development and its underlying neural mechanisms in animals and humans.

1.3.2 Anxiety conditioning in animals and humans

According to Fanselow's predator imminence model (1994), the context rather than a distinct cue seems to be relevant for the expression of the sustained feeling of anxiety. What is a context and how can we model anxiety in an experimental setting? Maren, Phan, and Liberzon (2013, p. 418) described a context as '*a set of circumstances around an event*'. Features of a context stay relatively stable and depend on the variation of components of the context (Maren et al., 2013; Nadel & Willner, 1980; Rudy, 2009). Most intuitive, a context is spatial, but other forms also include temporal, interoceptive, cognitive, social and cultural contexts (Bouton, 1993; Maren et al., 2013). A context can help with the interpretation of an event, e.g. if we smell burned material or fire, we might respond differently when we sense it being in a closed building or sitting beside a campfire. Therefore, the context is an obvious source of information.

One model to elicit anxiety is context conditioning (Grillon, 2008). To this end in animal experiments, a context (anxiety context, CTX+), mostly a cage, is paired with an US, e.g. an electric footshock (Myers & Gluck, 1994). Human differential context conditioning experiments add another context (safety context, CTX-) which is never paired with an US. Importantly, in context conditioning, the diffuse state of anxiety is elicited by presenting the US unpredictably during the presence of CTX+ (Grillon, 2008). In parallel to cue conditioning, context conditioning can be measured on genetic level (Heitland, Groenink, Bijlsma, Oosting & Baas, 2013), on physiological level including skin conductance level and startle response (Glotzbach-Schoon, Andreatta, Mühlberger & Pauli, 2015), on cortical level using ssVEPs (Kastner, Pauli & Wieser, 2015) as well as on subcortical level (Lang et al., 2009), subjective reports and behavior including avoidance (Glotzbach, Ewald, Andreatta, Pauli & Mühlberger, 2012). Studies in animals found the hippocampus and BNST, the extended amygdala, as key structures for context conditioning (Fanselow,

1990; Kim & Fanselow, 1992; Myers & Gluck, 1994; Rudy, Barrientos & O'Reilly, 2002). During contextual learning, the hippocampus is essential to integrate multiple single cues to one contextual representation (Fanselow, 1990; Moses, Winocur, Ryan & Moscovitch, 2007; Nadel & Willner, 1980). Notably, Fanselow (1990) described the context pre-exposure effect, which indicates the necessity of the creation of a spatial map of a context in order to successfully associate the immediate US with a context. Such hippocampal context representation is necessary to successfully perform context conditioning, as the unpredictable US is associated with the conjunctive contextual representation rather than single elements (Rudy, Huff & Matus-Amat, 2004). Supporting this, post-training lesions of the dorsal hippocampus caused retrograde deficits in anxiety. Here, the expression of anxiety is impaired because the information that was acquired by the hippocampal system is not available anymore (Fanselow, 1990; Maren, Aharonov & Fanselow, 1997). In contrast, pre-training lesions did not show any learning impairment which indicated that context-specific cues mediated anxiety response by an overtaking amygdala (Maren et al., 1997). Altogether, animal studies suggest a dual representation system of a context consisting of a conjunctive representation mediated by the hippocampus and an elemental representation mediated by the amygdala (Nadel & Willner, 1980). Both systems might be tightly connected and necessary for the acquisition of normal and pathological anxiety (Grillon, 2008).

In human anxiety conditioning studies, a long-lasting color presented on a computer screen (Armony & Dolan, 2001; Pohlack, Nees, Ruttorf, Schad & Flor, 2012) or a picture (Lonsdorf, Haaker & Kalisch, 2014b) or a virtual (Baas et al., 2004; Tröger, Ewald, Glotzbach, Pauli & Mühlberger, 2012) or a real (LaBar & Phelps, 2005) room have served as contextual stimuli. Rather than pre-exposure in a cage, human anxiety conditioning studies use a habituation or exploration phase, in which participants get to know the

context and researchers evaluate baseline measurements prior to the conditioning procedure (Andreatta, Leombruni, Glotzbach-Schoon, Pauli & Mühlberger, 2015b; Glotzbach et al., 2012; Lonsdorf et al., 2014b; Pohlack et al., 2012). Kastner et al. (2015) performed a context conditioning paradigm using two pictures of virtual rooms as contexts and investigated electrocortical processing. The ssVEP amplitude is a measure of attention (Keil et al., 2003). This amplitude increased in the anxiety context, which has been paired with the US, compared to the safety context, which has never been paired with the US. Therefore, the results indicated enhanced attention towards potential threats which seems even stable with a distraction from the context (Kastner et al., 2015). For an even more realistic implementation of threat learning in the laboratory, virtual reality (VR) environments become more and more important due to their ecological validity under highly controlled experimental conditions (Alvarez et al., 2008; Baas et al., 2004; for review see Bohil, Alicea & Biocca, 2011; Grillon, Baas, Cornwell & Johnson, 2006; Mühlberger et al., 2008). Additionally, the different levels of anxiety according to Lang (1995) can be modeled by anxiety conditioning in VR. For instance, Glotzbach et al. (2012) described VR as an appropriate tool for the investigation of conditioned anxiety regarding subjective ratings and behavior. First, they reported successful anxiety conditioning in terms of lower valence, higher arousal and anxiety ratings in CTX+ compared to CTX-, second participants' avoidance of CTX+ reflected the anxiety behavior of the participants which emphasizes the promising impact of VR on the translation of findings in healthy participants to anxiety patients and exposure-based therapy in VR. Moreover, psychophysiological investigations in anxiety conditioning revealed higher startle responses as well as electrodermal activity in the anxiety compared to the safety context (Glotzbach-Schoon, Andreatta, Mühlberger & Pauli, 2013a). In line with animal studies, neuroimaging studies in humans found the hippocampus, amygdala, and BNST as key

structures involved in context conditioning, indicated by greater activations of these regions in CTX+ compared to CTX- (Alvarez et al., 2008; Alvarez et al., 2011; Andreatta et al., 2015a; for review see Glenn, Risbrough, Simmons, Acheson & Stout, 2017). The impaired processing of contexts associated with impaired hippocampal functioning might be involved in the etiology of PTSD (Acheson, Gresack & Risbrough, 2012; Liberzon & Abelson, 2016). In order to understand these underlying mechanisms, many studies investigated the brain activations evoked by threatening contexts in healthy participants (Andreatta et al., 2015a) and PTSD patients (Steiger, Nees, Wicking, Lang & Flor, 2015) using contextual anxiety conditioning in fMRI. Interestingly, PTSD patients showed deteriorated subjective learning of contingencies between threatening context and aversive event and additionally had higher hippocampus activation required to learn the associations (Steiger et al., 2015). In sum, anxiety conditioning is a well-established model in animals and humans for the investigation of anxiety and its pathological abnormalities.

1.3.3 Cue in context conditioning

Modeling the development of fear and anxiety by cue and context conditioning only gives information about the pure forms of fear or anxiety and its related pathological characteristics. For cue conditioning, Grillon (2008) described the high relevance of a distinct CS rather than the context as the cue reliably predicts the US. In contrast, during context conditioning, single elements of the context might be less important than the context itself, because the context is the most reliable predictor for the US (Grillon, 2008). So far, the impact of single contextual elements in comparison to conjunctive contextual representation on the peculiarity of fear and anxiety is still unknown. However, assuming someone, for instance, has been involved in a traumatic car accident and developed PTSD, the person experienced a complex situation of contextual stimuli like the visual context of the car and the street but also cued stimuli like a loud bang or the distinct red color of the

opponent's car (Glenn et al., 2017). Investigations of such complex connections require further approaches rather than single cue or context conditioning, e.g. cue in context conditioning. In fact, the learned association of a cue and any threat is dependent on the context (Huff et al., 2011). Hence, in the experimental model single cues like geometric shapes (CS) were presented together with different contextual stimuli, e.g. pictures of a room (CTX; Marschner et al., 2008). Only the combination of one geometric shape (CS+) presented in one context (CTX+) predicted the occurrence of an US. In contrast, the presentation of CS+ in the safety context (CTX-) has not been paired with the US. Such context-dependent cue conditioning studies have been performed previously and found the US predictability of a stimulus as the relevant mediator for fear or anxiety (see Grillon, 2008). Vansteenwegen, Iberico, Vervliet, Marescau, and Hermans (2008) used the illumination of the experimental room as contexts, i.e. light on vs. off. Moreover, they presented two geometric shapes as CS on a computer screen. Though USs were administered in both contexts, one CS was reliably followed by an US in one context, whereas in the second context another CS was explicitly never paired with an US. Fear potentiated startle responses to the CS, which predicted the US, compared to the other demonstrated cue conditioning. Additionally, context conditioning was shown by larger startle responses during the context in which the CS was unpaired with the US, assuming high US predictability of the context, but low of the CS. Even in the cue in context paradigms, VR technology gains importance, as it provides the possibility to combine a well-controlled virtual context with distinct, well-controlled cues appropriate for this context (Baas et al., 2004; Huff et al., 2011; Indovina, Robbins, Núñez-Elizalde, Dunn & Bishop, 2011; Mühlberger et al., 2014). Therefore, Baas et al. (2004) combined two virtual contexts with colored light panels. During conditioning, the appearance of one panel (CS+) in the anxiety context (CTX+) predicted the US. No US was delivered neither during CS-

presentation in CTX+, nor during CS+ and CS- presentation during CTX-. Subsequently, after acquisition, startle response was potentiated in CTX+ compared to CTX-, and for CS+ compared to CS- in both contexts. Although participants explicitly learned the US contingencies, they generalized the stimuli across both contexts thereby proving that fear conditioning was indicated by the cues (Baas et al., 2004). In this line, Mühlberger et al. (2014) investigated contextual learning in genetic variants of the brain-derived neurotrophic factor (BDNF). Met+ carriers compared to homozygous Val carriers showed reduced BDNF concentrations in brain regions like hippocampus, amygdala and cerebral cortex which are involved in synaptic plasticity and long-term potentiation, learning and memory (Bramham & Messaoudi, 2005; Chiaruttini et al., 2009; Egan et al., 2003). Indeed, in their cue in context conditioning paradigm using virtual offices as contexts and colored lights as cues, carriers of both polymorphisms showed intact context-dependent cue conditioning in terms of potentiated startle responses to CS+, but not CS- in CTX+. However, Met+ carriers showed similar startle responses to both contexts (CTX+ and CTX-), whereas Val/Val carriers revealed potentiated startle responses to CTX+ compared to CTX-. From these results, Mühlberger et al. (2014) concluded that BDNF might be a risk factor for the development of anxiety disorders.

Another established possibility for a differential investigation of both fear and anxiety is the so-called NPU paradigm (Grillon et al., 2006; Schmitz & Grillon, 2012). Here, the predictability of the US is the main construct for anxiety (Foa, Zinbarg & Rothbaum, 1992). In this paradigm, three CS-CTX combinations are used: In the neutral condition (N), context and cue are presented without any US delivery. The predictable condition (P) contains a second context and US presentations are associated with a distinct cue that models fear. In the unpredictable condition (U), a third context and cue are used, and the US delivery happens only associated with the context, not with the cue, modeling anxiety.

Grillon et al. (2006) found successful fear conditioning in terms of potentiated startle responses to the CS in the predictable compared to the unpredictable and neutral conditions. Furthermore, they showed anxiety conditioning regarding potentiated startle responses and higher subjective anxiety in the context of the unpredictable condition compared to predictable and neutral. Even behavioral data indicated participants' preferences to re-enter the neutral followed by the predictable context compared to the unpredictable one after acquisition (Grillon et al., 2006). Therefore, the US signaled by the cue in the predictable context elicited fear of a distinct stimulus (light) rather than of the context. In contrast, anxiety was shown to the context, less to the cue, for the unpredictable condition as this is still the best predictor for an upcoming US (Schmitz & Grillon, 2012). Attentional mechanisms in phasic fear to the threat predicting cues, and in sustained anxiety to the uncertain dangerous context were investigated by Wieser, Reicherts, Juravle, and von Leupoldt (2016). They demonstrated larger electrocortical responses in terms of enlarged ssVEP amplitudes at the context onset of the unpredictable compared to the predictable condition as well as larger ssVEPs for the onset of cues in the predictable compared to the neutral condition. They interpreted the described attentional effects as showing enhanced processing of fear predicting cues and the hypervigilant state of anxiety during an unpredictable US presentation (Wieser et al., 2016).

In sum, cue in context conditioning is a powerful paradigm to investigate not only the pure characteristics of fear or anxiety with their pathological pendants of specific phobias and generalized anxiety disorders but also to enlighten more complex clusters of symptoms which share fear and anxiety characteristics like PTSD.

1.4 Investigations of extinction learning and return of fear and anxiety

1.4.1 Extinction learning and relapse of fear and anxiety

Is the conditioned stimulus presented alone without the reinforcing US for a number of trials, extinction occurs (Phelps et al., 2004). During fear extinction, the conditioned fear responses decrement by repeated CS presentation (Milad & Quirk, 2012). Importantly, extinction is highly context-dependent (Bouton & Bolles, 1979; Bouton, Westbrook, Corcoran & Maren, 2006; Vansteenwegen, 2005). In parallel to fear acquisition, the fear extinction process can also be dissected into three phases: acquisition, consolidation, and retrieval (Quirk & Mueller, 2008). For instance, Mueller and colleagues (Mueller, Panitz, Hermann & Pizzagalli, 2014; Mueller & Pizzagalli, 2016) performed fear conditioning experiments in which they used two stimuli as CS+ and two as CS- during conditioning. In the subsequent extinction phase, they presented only one of the CS+ and one of the CS- again without US presentation. A recall test one day or even one year later assessed differential fear responses for the non-extinguished CS+ and CS- compared to the extinguished CS+ and CS- in skin conductance (Mueller et al., 2014). In other words, differential fear responses, which had previously been learned during an acquisition phase, maintain present when no extinction learning occurred. The memory consolidation overnight between acquisition and extinction, and between extinction and retrieval test allows the formation of a distinct but still flexible memory trace which confers the ability of extinction recall (Huff, Hernandez, Blanding & LaBar, 2009; Maren & Chang, 2006).

The acquisition of an extinction memory is a new learning process of the CS-noUS association rather than the erasure of the initial CS-US association (Bouton, 1988; Bouton & Moody, 2004; Bouton & Swartzentruber, 1991). Considering the Pavlovian inhibitory

learning model, the fear memory, i.e. the CS-US association, which was built during fear conditioning, competes with the extinction memory, i.e. the CS-noUS association, since the unreinforced presentation of the CS elicits ambiguity of the stimulus (for review see Bouton, 2002, 2004; Bouton & Moody, 2004). Subsequently, the CS has two meanings, an excitatory meaning of CS-US association and an inhibitory meaning of the CS-noUS association. Evidence for these assumptions supplied the phenomena of relapse of fear after extinction, i.e. re-acquisition, spontaneous recovery, renewal and reinstatement (Bouton, 2002).

During re-acquisition, the CS is again paired with the US after fear extinction (Bouton, 2002). Thereby, the recovery of the fear response can be very fast, as the original fear memory is not erased by extinction. However, the pairing of the extinguished signal with the reinforcer still causes ambiguity which can be disentangled by the context (Bouton, 2002). In humans, Agren, Furmark, Eriksson, and Fredrikson (2012) found increased fear re-acquisition measured by SCR, when extinction took place outside of the reconsolidation window which was defined as 6 h compared to 10 min after the acquisition. Therefore, fear responses rely on the strengths of the competing CS-US and CS-noUS associations.

Spontaneous recovery was already described by Pavlov (1927). He found that the extinguished responses recovered after a certain period of time when the CS was presented again. Since extinction depends on the context (Bouton et al., 2006), the change of the temporal context (see Maren et al., 2013) might lead to a failure of retrieval of the extinction memory in another context (Bouton, 2002). Quirk (2002) varied the time interval between extinction and the test of spontaneous recovery in rats and found a relapse of the initial freezing response after 10 to 14 days. An additional re-extinction phase demonstrated faster extinction compared to the first extinction phase which

indicated the presence of the extinction memory yet (Quirk, 2002). But, when exactly is a new memory formed? Gershman, Jones, Norman, Monfils, and Niv (2013) rose this question and hypothesized that a slow extinction at a slight instead of an abrupt change of reinforcement rate would modify only the fear memory by small prediction errors rather than build a new extinction memory. Indeed, the slow extinction significantly reduced spontaneous recovery which was interpreted as caused by a modified fear memory instead of a newly created extinction memory. However, in clinical practice, this procedure might be questionable since it involves many therapeutic sessions, many clinicians, and is therefore very costly. Shiban et al. (2015) aimed to strengthen the extinction memory and investigated the influence of multiple context exposure on spontaneous recovery in spider phobic patients after exposure treatment in virtual reality. Interestingly, the use of multiple contexts for exposure therapy reduced the return of fear immediately after treatment. The exposure treatment in a single context with multiple stimuli, i.e. spiders, seemed to be more effective for the long-term memory, which was explained by the expectancy violation theory (reviewed in Craske, Treanor, Conway, Zbozinek & Vervliet, 2014). The return of fear in patients does not necessarily lead to fear relapse and relapse of clinical symptomatology (Vervliet, 2013). However, in the case of a spider phobic patient, even after exposure treatment the fear might return in terms of spontaneous recovery if a patient has not been exposed to a spider for a long time after therapy (Vervliet, 2013).

Further evidence that first, extinction is context-dependent, and second, the fear and the extinction memory compete, suggests the renewal effect. In detail, the extinguished fear might recover in a different context than the extinction context (Bouton, 2002; Bouton & Bolles, 1979). Several experimental designs containing acquisition, extinction and test phase investigate renewal effects. In an ABA design, acquisition takes place in

context A, extinction in context B, and test in context A. In such a design, Rauhut, Thomas, and Ayres (2001) found pronounced renewal effects in rats, which performed a short extinction phase and in rats which underwent five times more extinction trials suggesting a very stable effect of the context. In parallel, humans also showed renewal effects depicted in larger skin conductance responses and US expectancy ratings when they underwent fear conditioning in an ABA design compared to an AAA design (Vansteenwegen, 2005). However, when single cues that were shown in either the acquisition phase or the extinction phase, were presented in the retrieval test, SCR and US expectancy ratings were enhanced for participants that saw the acquisition cue compared to participants that saw the extinction cue (Vansteenwegen et al., 2006). Such cues might serve as reminders for fear or safety. In a similar design, Huff et al. (2009) investigated an ABA design in humans and found lower renewal and rapid re-extinction when extinction took place 24 h after fear acquisition compared to extinction directly after the acquisition. Regarding the spider phobic patient mentioned above, a therapist can train patients to catch a spider with a glass and release it into nature during a therapy session. However, the fear can return when the spider appears in the patient's home rather than at the therapy room (Vervliet, 2013).

Reinstatement is another fear relapse mechanism, which describes the return of fear after extinction learning. Here, the US is administered unannounced after extinction and in a different contextual setting (e.g. temporal or spatial) which can lead to a recovery of the initial fear responses (Bouton, 2002). Already Pavlov (1927) and Rescorla and Heth (1975) investigated reinstatement in animals. Likewise, reinstatement effects depend on the context: Is an animal exposed to an US, the context-independent CS-US association is retrieved and the fear memory is present compared to the CS-noUS association which depends strongly on the context (Bouton, 2004; but see Haaker, Golkar, Hermans &

Lonsdorf, 2014b for a review). Interestingly, Sokol and Lovibond (2012) found that reinstatement can also happen when a new US is used for reinstatement which has the same valence as the US during acquisition. In detail, they used a loud noise as the US for conditioning and an electric shock as reinstatement US and found the return of fear as in the classical one US paradigm in skin conductance response. Using two USs however, increased US expectancy ratings after reinstatement compared to one US paradigm which might indicate the implication of further cognitive learning processes involved in reinstatement. Haaker et al. (2014b) summarized the possible mechanisms underlying reinstatement. In detail, they propose the occurrence of a single mechanism or a compound of mechanisms including attention (Pearce & Hall, 1980), the attention-associative model (Schmajuk, Larrauri & LaBar, 2007), the associative chaining framework (Hall, 1996) and contextual learning (Westbrook, Iordanova, McNally, Richardson & Harris, 2002) dependent of the experimental design. Human studies frequently use protocols of differential conditioning in order to investigate reinstatement effects. Here, the US for reinstatement is presented and therefore recalled while the screen on which CS+ and CS- were presented is black. Some studies found differential reinstatement meaning return of fear for CS+ but not for CS- (Dirikx, Hermans, Vansteenwegen, Baeyens & Eelen, 2007; LaBar & Phelps, 2005; Norrholm et al., 2006), others found generalized reinstatement meaning return of fear for both CS+ and CS- (Dirikx, Vansteenwegen, Eelen & Hermans, 2009; Kull, Müller, Blechert, Wilhelm & Michael, 2012). The same is true for context conditioning paradigms: Differential anxiety conditioning was found in Glotzbach-Schoon et al. (2015), who showed differential reinstatement by increased anxiety ratings and potentiated startle responses in CTX+ but not in CTX-. Generalized reinstatement to their contextual stimuli could be found by Haaker, Lonsdorf, Thanellou, and Kalisch (2013) indicated by SCR and startle response

and partially by fear ratings. Generalized reinstatement can be caused by sensitization and orientation to uncertainty (Haaker et al., 2013). Several studies included a control group who had a small break rather than reinstatement and found return of fear in this group as well which could be explained by the break causing spontaneous recovery (Dirikx et al., 2007; Haaker et al., 2014b; Hermans, Craske, Mineka & Lovibond, 2006; Kull et al., 2012). Another even more important explanation for generalized reinstatement is stimulus generalization, i.e. similarities of CS+ and CS- which leads to impaired safety learning (Lissek et al., 2005). The impairment of the discrimination of threat and safety cues is associated with pathological anxiety (Duits et al., 2015; Lissek et al., 2005). Vervliet (2013) described the example of a person who experienced a panic attack in an elevator and consequently is now afraid of elevators and avoids them completely. After cognitive-behavioral treatment, he is able to enter elevators again with only little discomfort. However, if he again experienced a panic attack unrelated to any elevator, the fear might have been reinstated and he might relapse into his anxiety disorder (Vervliet, 2013). In sum, reinstatement is a clinically very relevant mechanism to understand the underlying mechanisms, to maximize exposure therapy outcome, and to prevent fear relapse due to elevated arousal levels or negative associations to the feared object or situation. As previous findings in experimental settings are very heterogeneous, the return of fear and anxiety needs further investigations.

1.4.2 The neural correlates of extinction

The translation of animal findings, especially in rodents, to humans allows some indications of the mechanisms of extinction learning and its relevant brain regions. The amygdala is not only important during fear acquisition, but also in fear extinction (Dunsmoor, Niv, Daw & Phelps, 2015; Myers & Davis, 2002; Myers & Davis, 2007).

Multiple potential circuits for fear extinction have been suggested with the subsequent underlying pathways: The lateral amygdala indirectly projects via the basal nucleus and intercalated cells of inhibitory gamma-aminobutyric acid (GABA-ergic) neurons to the central nucleus of the amygdala; the basal nucleus projects directly to intercalated cells (Dunsmoor et al., 2015; Hartley & Phelps, 2009). Information from the infralimbic cortex (IL) in rats or the ventromedial prefrontal cortex (vmPFC) in humans activates the intercalated cells either directly or indirectly via the basal nucleus (for a review see VanElzakker, Dahlgren, Davis, Dubois & Shin, 2014). Within the central nucleus of the amygdala, the centrolateral subdivision inhibits the centromedial subdivision which in turn inhibits the performance of the fear response (VanElzakker et al., 2014). Morphological changes of synaptic plasticity in the BLA were shown to facilitate the consolidation of extinction (Chhatwal, Myers, Ressler & Davis, 2005). Pharmacological manipulations demonstrated that blockade of glutamate receptors in the amygdala impaired extinction (Kim et al., 2007; Sotres-Bayon, Bush & LeDoux, 2007).

Gewirtz, Falls, and Davis (1997) found the rodent mPFC, more specifically the infralimbic subregion of the mPFC, as highly relevant for the inhibition of conditioned fear responses and the vmPFC directly projects to the amygdala (McDonald, 1998). Milad and Quirk (2012) summarized in their review the infralimbic cortex in rodents as the homologous structure to the vmPFC in humans (Kalisch et al., 2006; Phelps et al., 2004). Lesions of the vmPFC induce impaired fear extinction and retention of extinction (Morgan & LeDoux, 1995; Morgan, Romanski & LeDoux, 1993; Quirk, Russo, Barron & Lebron, 2000; Sotres-Bayon & Quirk, 2010). Initial imaging studies found increased amygdala and orbitofrontal cortex activation during extinction training (Gottfried & Dolan, 2004; Knight, Smith, Cheng, Stein & Helmstetter, 2004). The infralimbic activity has been

suggested to boost the consolidation of extinction and subsequently inhibit fear expression when facing a threatening stimulus (Milad & Quirk, 2002).

Furthermore, the high context-dependency of the extinction information and the competition between the original fear memory and the new extinction memory (Bouton, 2004) suggests the hippocampus as a key structure for fear and anxiety extinction. Indeed, Maren et al. (2013) described the hippocampus as a control region for context-specific retrieval of extinction, which is mediated through indirect projections to the vmPFC and direct projections to the lateral amygdala.

In sum, rodent work showed the interaction between the amygdala, vmPFC, and hippocampus as a central structure in the acquisition, retrieval and contextual modulation of fear extinction (Dunsmoor et al., 2015; Milad & Quirk, 2012). This is especially interesting due to the translation of findings in humans to rodents. Imaging studies in humans provide evidence for preservation of learning mechanisms: The hippocampus, in particular the anterior part, was found to contribute to the expression of the extinction memory (Kalisch et al., 2006; Milad et al., 2009; Milad et al., 2007) and the posterior hippocampus for the return of fear cues (Kalisch et al., 2009; Kalisch et al., 2006). Gottfried and Dolan (2004) performed an aversive olfactory conditioning and extinction experiment using fMRI and found the orbitofrontal cortex as well as lateral amygdala activity during extinction. The lateral amygdala processes changing contingencies of CS-US relationships, as it is active during both acquisition and extinction (Hartley & Phelps, 2009; Knight et al., 2004). However, the most important brain region in this context is the vmPFC. Neural connections from the hippocampus to vmPFC integrate relevant contextual information for extinction learning and memory recall (Kalisch et al., 2006; Milad et al., 2007). The activation of the vmPFC increases especially during recall of extinction memory (Kalisch et al., 2006; Phelps et al., 2004). The thickness

of vmPFC regions in humans correlated with extinction memory recall, which is suggested to explain individual differences in fear expression and the modulation of fear recall (Hartley, Fischl & Phelps, 2011; Milad et al., 2005). Lonsdorf et al. (2014b) investigated the brain regions involved in the processing of reinstatement. They performed an aversive conditioning experiment and measured fMRI after reinstatement and found vmPFC activation for extinction recall besides increasing the amygdala and anterior hippocampus activity after reinstatement. To conclude, for both cue and contextual CSs amygdala, hippocampus and vmPFC are the main structures for the mediation of extinction recall and return of fear in humans and starting points for promising interventions in psychotherapy.

1.4.3 Experimental approaches on extinction and relapse

Several experimental and clinical approaches have already been investigated on how to facilitate extinction and prevent fear and anxiety relapses (Craske, Hermans & Vervliet, 2018). Vervliet et al. (2013) discussed many of them in their review: prolonged extinction training enhanced extinction learning (Alvarez-Dieppa, Griffin, Cavalier & McIntyre, 2016) and reduced renewal effects in rats (Denniston, Chang & Miller, 2003); compound extinction also attenuated spontaneous recovery and reinstatement in rats, but not in humans (Rescorla, 2006; Vervliet, Vansteenwegen, Hermans & Eelen, 2007); an additional cue to extinction training as retrieval cue can turn into a safety signal (Dibbets & Maes, 2011), but the absence of this safety reminder might boost return of fear during CS presentation (Brooks & Bouton, 1994); mental reinstatement of the treatment episode reduced renewal in participants with fear of spiders (Mystkowski, Craske, Echiverri & Labus, 2006); multiple contexts under some circumstances reduced reinstatement or renewal (Dunsmoor, Ahs, Zielinski & LaBar, 2014; Vansteenwegen et al., 2007), e.g.

operationalized by different background colors of the screen during extinction reduced renewal in spider phobics; the extinction of multiple stimuli, e.g. multiple spiders in spider phobics, might also reduce return of fear (Rowe & Craske, 1998; Shiban et al., 2015); the devaluation of the US by imagery rescripting reduced renewal in humans (Dibbets, Poort & Arntz, 2012). Additionally, cognitive enhancers can augment the consolidation of extinction memory during extinction training or exposure sessions during cognitive behavioral therapy in anxiety patients. D-cycloserine is a partial N-methyl-D-aspartate (NMDA) agonist and primarily involved in NMDA mediated plasticity (Myers & Davis, 2007) and formation and consolidation of extinction memory (Richardson, Ledgerwood & Cranney, 2004). Infusions of D-cycloserine in rats enhanced long term extinction assessed by startle responses (Walker, Ressler, Lu & Davis, 2002). However, some phenomena linked with the return of fear like renewal and rapid reacquisition stayed intact even after D-cycloserine intake (Ledgerwood, Richardson & Cranney, 2004; Woods & Bouton, 2006). In humans, the addition of D-cycloserine to height exposure therapy enhanced long term extinction effects in acrophobia patients (Ressler et al., 2004). Though D-cycloserine is the best understood cognitive enhancer for the treatment of anxiety disorders, there are many studies investigating others including cortisol, catecholamines, yohimbine, caffeine (for a review see Hofmann, Smits, Asnaani, Gutner & Otto, 2011). However, some cognition-enhancing drugs have disadvantages like the anxiogenic effects of the α_2 adrenergic receptor antagonist yohimbine (Cain, Blouin & Barad, 2004).

Another approach for manipulation of extinction learning is stress, in particular, the cortisol level in organisms (reviewed in Maren & Holmes, 2016), because stress influences acquisition and retrieval of extinction in humans (Maren & Holmes, 2016; Raio & Phelps, 2015). Hartley, Gorun, Reddan, Ramirez, and Phelps (2014) stressed their participants

and performed fear conditioning, extinction, and retrieval test a few days later. Interestingly, extinction retrieval assessed by skin conductance response was enhanced when participants could avoid the stressor. However, extinction was impaired when participants could not escape the stressor (Hartley et al., 2014). In case of stress exposure before the test of retrieval, the return of the conditioned response was assessed in several studies (Merz, Hamacher-Dang & Wolf, 2014; Raio, Brignoni-Perez, Goldman & Phelps, 2014). Kinner, Merz, Lissek, and Wolf (2016) administered cortisol to their healthy participants before extinction retrieval test. They found first, behaviorally impaired extinction retrieval by renewal compared to a placebo control group. Second, reduced context differentiation was revealed for the extinguished stimulus in vmPFC which the authors interpreted as disruption of vmPFC functioning to communicate with other brain areas that are involved in extinction. In parallel, PTSD accompanied by stress not only impairs the recovery from trauma due to impaired fear extinction but might also hinder the attenuation of fear by inhibitory circuits, which again involves the amygdala, prefrontal cortex, and hippocampus (Maren & Holmes, 2016).

The arousal level of an organism is another key player that modulated fear acquisition and extinction processes and is currently under investigation through many different approaches. During arousal, catecholamines including dopamine (DA) and norepinephrine (NE), which are counted among the most important neurotransmitters in the central nervous system, are released to form adaptive memories of an important event (Harley, 2004). Giustino and Maren (2018) reviewed these memory processes mainly in animal studies and displayed its dependency on the locus coeruleus-norepinephrine (LC-NE) system. Noradrenergic β-receptors in the brain, in particular in the IL in rats, are activated by NE and mediate a strong extinction memory (Mueller, Porter & Quirk, 2008). A dysregulation of NE in memory and retrieval was demonstrated in many studies

investigating PTSD (Giustino, Fitzgerald & Maren, 2016; Raio & Phelps, 2015). Giustino and Maren (2018) suggest the modulation of learning through the state of behavioral arousal and therefore through the LC-NE system which is suggested to modulate the mPFC via an inverted U-shape curve (Arnsten, 2009, 2015; Arnsten & Li, 2005). High levels of stress and arousal promote BLA activity and reduce mPFC functioning leading to enhanced fear acquisition (Giustino & Maren, 2018). On the other hand, low levels of arousal at the onset of extinction learning (Maren & Holmes, 2016) lead to higher PFC functioning and inhibition of downstream signaling to the BLA leading to improved extinction learning (Giustino & Maren, 2018). Besides fear conditioning, the LC-NE system is also important for the development of context conditioning and extinction processes. Contextual information is encoded in the hippocampus, which has a dense adrenoceptor expression, and NE is able to regulate long-term potentiation (Giustino & Maren, 2018). Furthermore, the connectivity between the hippocampus and mPFC is affected by NE in the BLA and promotes contextual fear learning (Giustino & Maren, 2018). Interestingly, a study in rats by Do-Monte et al. (2010) found that injection of the β-adrenoreceptor agonist isoproterenol into the vmPFC prior to contextual extinction learning facilitated extinction, whereas blocking of β-adreno-receptors by propranolol impaired contextual extinction (Mueller et al., 2008). Therefore, the translation of these findings on the modulation of noradrenergic mechanisms by drugs is of great interest for the treatment of PTSD patients (for a review see Fitzgerald, Seemann & Maren, 2014). Recently, a study with healthy human volunteers found momentarily increased arousal by threatening stimuli which lead to increased LC activity and stronger memory formation (Clewett, Huang, Velasco, Lee & Mather, 2018). The moderate activation of the LC-NE system indeed facilitates memory formation, including extinction learning (Arnsten, 2015; Arnsten, Raskind, Taylor & Connor, 2015; Sara, 2015). However, a high level of arousal, resulting

in stress, impairs extinction learning and fosters fear generalization (Dunsmoor, Otto & Phelps, 2017; Maren & Holmes, 2016; Raio & Phelps, 2015). In this line, yohimbine, a noradrenergic receptor agonist, was tested in healthy participants and was found to strengthen fear learning and impair extinction learning (Soeter & Kindt, 2011; van Stegeren, Roozendaal, Kindt, Wolf & Joëls, 2010). In contrast, human studies investigating noradrenergic receptor antagonists like propranolol found reduced long-term memory of emotional stories (Cahill, Prins, Weber & McGaugh, 1994), reduced emotional arousal in subjective ratings and reduced skin conductance level in context conditioning (Grillon, Cordova, Morgan, Charney & Davis, 2004), but also no effects neither on acquisition nor retention of fear extinction (Nugent et al., 2010; Orr et al., 2006). Giustino and Maren emphasized the timing of propranolol administration and argued for more research to overcome these obstacles of diverging results on the LC-NE system and memory consolidation in human studies (Giustino et al., 2016; Giustino & Maren, 2018).

Brain stimulation techniques like transcranial direct current stimulation (tDCS) or transcranial magnetic stimulation (TMS) are additional experimental approaches to modulate extinction learning and stabilize extinction memory in regard to return of fear and anxiety (Asthana et al., 2013; Guhn et al., 2014; van 't Wout et al., 2016). However, most importantly concerning the LC-NE system in humans, one new non-invasive stimulation technique is very promising to facilitate fear extinction and prevent relapse: transcutaneous vagus nerve stimulation (tVNS).

In sum, a lot of research has been performed on various approaches to understand and facilitate extinction learning and relapse mechanisms in animals and humans. However, results are inconsistent in the establishment of the optimal method for extinction learning and in the translation of animal studies to healthy humans and patients. Some studies hold great promise for implications into therapy. However, considering recent findings, more

research is needed to understand underlying mechanisms, find multi-level approaches of therapy and adapt therapy to individual differences for an optimal outcome.

1.5 Vagus nerve stimulation

In the 19th century, James L. Corning, a neurologist from New York, who studied the mechanisms of epileptic seizures, detected the manual massage and compression of the carotid artery in the cervical region of the neck as a successful treatment (Lanska, 2002). Though his innovative therapy held great promise, the side effects like dizziness and syncope were tremendous. Interestingly, Zanchetti, Wang, and Moruzzi (1952) stimulated afferent vagus nerve fibers in cats and recorded EEG simultaneously. They found changes in systemic blood pressure, EEG desynchronization by 2-300 Hz stimulation frequency and the suppression of epileptic activity depending on stimulation frequency. In particular, Chase, Sterman, and Clemente (1966) found EEG desynchronization following a stimulation frequency lower than 70 Hz, whereas higher stimulation frequencies induce EEG synchronization. Later, Jacob Zabara also raised the vagus nerve as a pathway to control seizures (Zabara, 1992). Only 1980, the first vagus nerve stimulator was successfully implanted in a young epilepsy patient and reduced his seizure rate by about 80% (Vonck et al., 2014). The Federal Food and Drug Administration approved VNS for the prevention of seizures in patients suffering from drug-resistant epilepsy in 1997 (e.g. in Noble et al., 2017). Eight years later, 20,000 epilepsy patients were already treated with an implanted VNS, pointing out a response rate of 50% (Vonck et al., 2005). Up to now, vagus nerve stimulation (VNS) is an established tool for the intervention of medically intractable epilepsy (Couch, Gilman & Doyle, 2016; Krahl & Clark, 2012). Though the exact mechanisms of action are unclear, desynchronization of neural activity, increased alertness and neurotransmitter regulation of NE, serotonin (5-HT) or dopamine (DA)

have been suggested (Krahl & Clark, 2012; Nasrin & Alireza, 2016). Notably, VNS is also a promising method for the treatment of various disorders including migraine, depression and Alzheimer's disease (for a review see Beekwilder & Beems, 2010; Cimpianu, Strube, Falkai, Palm & Hasan, 2017).

1.5.1 Anatomy and Physiology

The *Nervus vagus* is the 10th cranial nerve. It consists of small-diameter unmyelinated C-fibers transmitting afferent visceral and motor input, intermediate diameter myelinated B-fibers transporting parasympathetic information and large diameter myelinated A-fibers carrying afferent visceral input (Ruffoli et al., 2011; Schachter & Saper, 1998). Notably, the activation of A- and B-fibers requires low levels of current delivery by VNS (between 0.02 and 0.2 mA and between 0.04 and 0.6 mA, respectively; Groves & Brown, 2005), whereas C-fibers need currents of more than 2.0 mA to be activated (Groves & Brown, 2005; Ruffoli et al., 2011). The vagus – the *wandering* – nerve is a mixed nerve consisting of 20% efferent and 80% afferent fibers (Agostoni, Chinnock, Daly & Murray, 1957; Foley & DuBois, 1937). The efferent fibers form parasympathetic innervations to lungs, heart, gastrointestinal tract, larynx and pharynx (Ben-Menachem, 2002; Howland, 2014). Interestingly, the innervation of the heart was found to be asymmetric: While the right vagus nerve innervates the sinoatrial node, the left vagus innervates the atrioventricular node (Ben-Menachem, 2002; Saper et al., 1990). This was a very important finding because the innervation of the arteria seems to be denser compared to that of the ventricles and therefore the likelihood of causing cardiac effects is lower by stimulating the left compared to the right side of the vagus nerve (Schachter & Saper, 1998). In other words, the risk for bradycardia is lower by stimulating the left compared to the right vagus nerve which is the reason for the standard stimulation of the left vagus. The afferent fibers emanate from inner organs like lungs, heart, gastrointestinal

tract and aortic chemoreceptors, as well as most important for the studies of this dissertation, from the concha of the ear (Ben-Menachem, 2002). Interestingly, when Klarer et al. (2014) cut the abdominal vagal afferents in rats, they found generally reduced anxiety-like behaviors as well as increased fear conditioning and attenuated extinction learning. They concluded that changes in neurotransmitter concentrations of NE and GABA in the limbic system of the brain rather than the hypothalamus-pituitary-adrenal gland (HPA) axis may modulate fear (Klarer et al., 2014). Besides, primarily viscerosensory information is transduced to four nuclei in the brain medulla (Ruffoli et al., 2011): the spinal nucleus of the trigeminal nerve, the nucleus ambiguous, the dorsal nucleus of the vagal nerve and the nucleus of the solitary tract (NTS), which represents the main portion of vagal afferents (Contreras, Beckstead & Norgren, 1982; Nemeroff et al., 2006; Schachter & Saper, 1998). One pathway via medulla and spinal cord triggers the baroreceptor reflex by slowing down the heart rate when the blood pressure increases and causes the end of respiration when the lungs are stretched (Schachter & Saper, 1998). Another important pathway from NTS leads via both excitatory and inhibitory noradrenergic dendrites to the locus coeruleus (LC; Van Bockstaele, Peoples & Telegan, 1999). Then, further noradrenergic connections ascend to hippocampus, thalamus and insular cortex, as well as hypothalamus, amygdala, dorsal raphe nucleus, cerebellum and cerebral cortex (Jones & Moore, 1977; Mason & Fibiger, 1979; Sakai, Touret, Salvert, Leger & Jouvet, 1977; Schachter & Saper, 1998).

Until now, the exact physiological mechanisms of VNS are still unknown (Childs, Alvarez-Dieppa, McIntyre & Kroener, 2015). Childs et al. (2015) described brainstem innervations and higher-order projections of the vagus nerve in rats. Among others, serotonin (Manta, Dong, Debonnel & Blier, 2009), GABA and NE (Ben-Menachem et al., 1995) are the most relevant neurotransmitters released during vagal activation.

GABAergic projection from PFC to the amygdala might inhibit conditioned fear responses (Childs et al., 2015; for a review see Makkar, Zhang & Cranney, 2010). NE in forebrain areas plays an important role in memory formation and consolidation, particularly during fear extinction (Çalışkan & Albrecht, 2013; Quirk & Mueller, 2008).

More precisely, emotional arousal can modulate long-term memory due to higher salience as well as physiological changes in the brain (McIntyre, McGaugh & Williams, 2012). As described by Fanselow (2013), we all remember easily what we have done on September 11th in 2001, while we might not remember what we have done on September 11th last year. In a state of excitement, peripheral epinephrine and glucocorticoids like cortisol are released from the adrenal glands (Segal & Cahill, 2009). Subsequently, epinephrine binds to vagal β-adrenergic receptors which stimulate the vagus nerve and finally increases NE release in the brain (Miyashita & Williams, 2006; Segal & Cahill, 2009). When stimulating the left vagus invasively, NE concentration in the brain rises instantaneously (Raedt et al., 2011; Roosevelt, Smith, Clough, Jensen & Browning, 2006). A high stimulation rate results in the activation of many vagal nerve fibers, which consequently increases the firing rate of LC neurons and the NE concentrations in the hippocampus and cortex (Dorr & Debonnel, 2006; Roosevelt et al., 2006). The importance of the LC-NE system on fear acquisition and extinction has already been emphasized earlier (Giustino & Maren, 2018). Key structures of emotional memory formation including the amygdala, hippocampus and vmPFC are activated by NE (Hassert, Miyashita & Williams, 2004; Roosevelt et al., 2006), either directly via the NTS or indirectly via the LC (Mello-Carpes & Izquierdo, 2013; Van Bockstaele et al., 1999). The injection of NE directly into the amygdala of rats lead to enhanced extinction learning (Berlau & McGaugh, 2006) which indicated the imitation of a peripheral adrenergic activation, i.e. the state of arousal (Fanselow, 2013). In this line, the release of NE and the increase of

synaptic plasticity may facilitate long-term potentiation (LTP) and therefore memory formation, in particular in hippocampus and PFC (Hopkins & Johnston, 1988; Miyashita & Williams, 2006; Mueller et al., 2008; Vonck et al., 2014).

For most epilepsy patients, electrodes implanted along the cervical portion of the nerve implemented stimulation of the vagus. Recently, a non-invasive VNS technique has been developed, which makes use of the fact that the cymba conchae of the human external ear is innervated exclusively by the auricular branch of the vagus nerve (ABVN; Peuker & Filler, 2002). The ABVN has the same ratio of afferent myelinated A beta axons as the cervical vagus nerve (CVN) which indicates similar effects of transcutaneous vagus nerve stimulation (tVNS) via the ear (Safi, Ellrich & Neuhuber, 2016). Although the ABVN relates somatosensory rather than viscerosensory information to the brainstem, its central projections reach the NTS (Nomura, 1984) and recent evidence documents that transcutaneous stimulation of this area leads to widespread changes in the activation states of the NTS and of other primary and higher-order targets of vagal sensory information in brainstem and forebrain (Frangos, Ellrich & Komisaruk, 2015).

1.5.2 Vagus nerve stimulation, fear extinction, and retrieval

Vagus nerve stimulation might be a promising method to activate the crucial brain network involved in the formation and consolidation of extinction memory (Beekwilder & Beems, 2010). VNS may thus be regarded as a tool to enhance the communication between the periphery and the central nervous system (CNS) during extinction learning (Çalışkan & Albrecht, 2013; Fischer, Ventura-Bort, Hamm & Weymar, 2018). Indeed, VNS-induced facilitation of extinction learning was supported by Peña et al. (2013), who performed a conditioning experiment in rats applying electric foot shocks during a 30 s lasting tone. Subsequently, the tone elicited sustained fear. In this single cue conditioning

experiment, they demonstrated faster extinction and less return of conditioned freezing in rats, which received vagus nerve stimulation via implanted electrodes during extinction compared to sham stimulated rats. Strikingly, the effect was also observed when the extinction treatment took place two weeks after the fear acquisition, emphasizing the unspecific requirements for a time window for treatment (Fanselow, 2013; Peña et al., 2013). In their follow-up study, Peña et al. (2014) also demonstrated similar results for the extinction of context conditioned anxiety indicated by decreased freezing behavior in VNS compared to sham stimulated rats when exposed to the US associated cage. The IL-BLA pathway is presumably the most important area for the formation and expression of the extinction memory (Childs et al., 2015). Peña et al. (2014) and Alvarez-Dieppa et al. (2016) investigated the biological mechanisms of VNS by induced long-term potentiation in rats' IL-BLA pathway. They found enhanced LTP in the BLA after high-frequency stimulation of the IL only in VNS treated rats during extinction, but neither in sham-treated rats nor in rats that received prolonged extinction training. Interestingly, the VNS effects disappeared when extinction memory consolidation was blocked directly after extinction training suggesting a natural extinction process accelerated by VNS. Recently, Noble et al. (2017) discussed possible mechanisms for successful PTSD treatment in a rat model that showed extinction learning and attenuated reinstatement compared to sham stimulated animals. Importantly, the enhanced neural plasticity through the release of neurotransmitters into the brain by VNS is experience-dependent. Therefore, the pairing of VNS with a sensory stimulus like auditory or motor learning increases plasticity specifically in the auditory or motor cortex, respectively (Hassert et al., 2004; Kilgard, 2012; Manta et al., 2009; Noble et al., 2017; Peña et al., 2014; Roosevelt et al., 2006). Hence, VNS during extinction in rats increases neural plasticity from infralimbic PFC to BLA complex (Alvarez-Dieppa et al., 2016; Peña et al., 2014). As

reported above, vmPFC activity is decreased and amygdala activity is increased in PTSD patients (Jovanovic et al., 2012; Norrholm et al., 2011). Hence, tVNS during extinction in PTSD patients might facilitate neural plasticity, strengthen the memory consolidation and therefore accelerate and stabilize extinction memory. So far, few studies have been performed in healthy humans and in anxiety patients and results were inconclusive. Fear conditioning studies proved extinction facilitating effects of tVNS in healthy humans, however only on the subjective level of contingency ratings (Burger et al., 2017; Burger et al., 2016). George et al. (2008) examined among others anxiety disorder patients, who were resistant to conventional treatment and received an implanted vagus nerve stimulator. They found improvement in anxiety symptoms in one-third of the patients suffering from obsessive-compulsive disorder (OCD), panic disorder (PD) or post-traumatic stress disorder (PTSD). However, the small sample size and the heterogeneity of disorders exacerbate the discussion of these results. Further research is needed to elucidate the effects of tVNS and subsequently use tVNS as an add-on treatment in exposure therapy in anxiety patients in the future.

1.6 Research Questions

The overall goal of this thesis is two-fold: first, the acquisition of fear and anxiety are investigated by the disentanglement of the dual representation of a context in order to shed light on the associative learning mechanisms that are involved in the development of conditioned fear and anxiety. Second, the extinction learning of fear and anxiety are investigated in healthy humans evaluating tVNS as a promising new tool for the facilitation as well as stabilization of extinction memory.

At first, I concurrently investigate the conjunctive and elemental representation of a context in the human brain during fear and anxiety acquisition. Thereby, I hypothesize a

strong interplay of both context representations during conditioning, which requires a fine dissociation of elemental and conjunctive context learning in conditioning studies (Study 1). Next, I focus on extinction and translate existing animal research on facilitated extinction learning by VNS to humans using tVNS. Therefore, I adapt a context conditioning design in virtual reality in order to examine tVNS effects on extinction and return of fear in humans and hypothesize facilitated and more stable extinction by tVNS in parallel to animal studies (Study 2). Due to the lack of reliable manipulation checks for tVNS, the replication of prior tVNS results is necessary. I, therefore, aim to replicate the analgesic effects induced by tVNS in electric pain and pressure pain stimulation (Study 3). Evidence for tVNS effects by prolonged stimulation and intermixed results in tVNS literature, considering both cue and context conditioning, lead to Study 4. Here I use a cue in context conditioning paradigm including context generalization in order to investigate the effects of prolonged tVNS on cue in context extinction and return of fear. I expect accelerated extinction learning, less return of fear and less fear generalization by tVNS compared to sham stimulated participants.

2 Study 1: The flip-book Experiment

This study has been submitted for publication (Genheimer, Andreatta, Pauli).

2.1 Introduction

The context, in which an event occurs, facilitates its interpretation and helps the organism to respond properly on neurobiological, behavioral and cognitive levels (Glenn et al., 2017). According to Bouton (1988) and later to Maren et al. (2013), a context is a set of continuously present circumstances, which includes spatial, temporal, cognitive, social, and cultural information. Particularly in threatening situations, effective processing of contextual information seems to be essential for survival.

Rudy and O'Reilly (2001) and Rudy et al. (2004) described the dual-process theory of a context in animals. In particular, a context can be represented as a set of single elements and features, which might, for instance, be the experimental chamber, the color of the wall or the light. This feature view (elemental representation) comprises independent elements that can per se be associated with an event. In contrast, the conjunctive representation of a context binds the separated elements into one unique representation and encodes the elements in a conjunctive view (conjunctive representation; Rudy, 2009; Rudy et al., 2004). While for the elemental representation the neocortical system (Nadel & Willner, 1980) and the amygdala (LaBar et al., 1998; Phillips & LeDoux, 1992) are involved in the associative learning of single elements and events in animals and in humans, the conjunctive representation relies highly on the involvement of the

hippocampus which integrates the elements into a conjunctive representation (Baeuchl, Meyer, Hoppstädter, Diener & Flor, 2015; Myers & Gluck, 1994; Rudy et al., 2004; Stout et al., 2018; Sutherland & Rudy, 1989).

For investigations of the dual-process theory and its underlying neural mechanisms in humans, fear and anxiety conditioning paradigms were used. So far, few studies investigated the conjunctive and elemental representation of the context during contextual fear conditioning and results are inconclusive. Baeuchl et al. (2015) developed a paradigm in which contexts were defined by the different placement of the elements, i.e. the furniture. Apparently, they revealed that this conjunctive information elicited anxiety responses in participants to that specific context configuration which has previously been paired with an aversive event compared to such configurations, which have never been paired with the US. Conditioned anxiety responses included decreased valence and enhanced arousal and contingency ratings as well as increased skin conductance level (SCL). However, the paradigm did not allow any conclusions on the elemental representation of the contexts. To solve this problem, Stout et al. (2018) investigated contextual anxiety learning by separate conjunctive and elemental learning paradigms. FMRI revealed hippocampus, but not amygdala activity in response to conjunctive context learning, and amygdala, but not hippocampus activity in response to elemental context learning. Fanselow (2000) found that the two systems compete but the conjunctive representation prevails in an intact brain (see also Glenn et al., 2017). However, impaired hippocampal functions prior to the processing of a context mean that the elemental representation, in particular, the amygdala, overtakes context processing (Maren et al., 1997; Stout et al., 2018). Importantly, the impaired processing of contexts associated with impaired hippocampal functioning might be involved in the etiology of PTSD (Acheson et al., 2012; Liberzon & Abelson, 2016). In order to understand these underlying

mechanisms, many studies investigated the brain activations evoked by threatening contexts in healthy participants (Andreatta et al., 2015a) and PTSD patients (Steiger et al., 2015) using contextual fear conditioning in fMRI. Interestingly, PTSD patients required higher hippocampal activation to learn associations between an anxiety context and an aversive event and additionally had difficulties in learning subjective contingencies (Steiger et al., 2015). These results emphasize the importance of investigations on the disentanglement of elemental and conjunctive context representations. A study, which integrates conjunctive and elemental representation of a context within the same experiment, is still missing. Moreover, fMRI studies lack the temporal resolution of fast stimulus processing. Therefore, a paradigm is required, which assesses quick responses to single elements of a context but simultaneously records ongoing responses to conjunctive context presentations. One solution for this problem might be the combination of context conditioning in virtual reality (Glotzbach-Schoon et al., 2013a) with multiCS conditioning (Steinberg, Bröckelmann, Rehbein, Dobel & Junghöfer, 2013). More precisely, Bröckelmann et al. (2011) and Steinberg et al. (2013) published an appropriate paradigm, i.e. multiCS conditioning which investigates physiological responses of few trial learning in humans without contingency awareness. Steinberg et al. (2012) paired 208 different pictures of 104 Caucasian faces with frontal and lateral view respectively with either pleasant (CS-) or unpleasant (CS+) odor. In a second experiment (Steinberg et al., 2013), the 104 frontal view faces were presented three times and either always paired (CS+) or never paired (CS-) with an electric shock while magnetoencephalography (MEG) was recorded. Interestingly, only two or three CS-US pairings respectively were required to show an increase in early or mid-latency neural activity over frontal and right occipito-parieto-temporal regions for CS+ compared to CS- stimuli after conditioning (Steinberg et al., 2013). Notably, the same was true for a

paradigm using faces as CS and startle probes as US (Steinberg et al., 2013) and for auditory fear conditioning (Bröckelmann et al., 2011). Although the modality of CS and US differed between studies, all participants were unaware of CS-US contingencies (Steinberg et al., 2013). Interestingly, physiological data indicated the differentiation between CS+ and CS- already in very early stages (< 100 ms) of stimulus processing (Steinberg et al., 2013). Visual stimuli can conceptually be processed very rapidly in humans (Potter, Staub, Rado & O'Connor, 2002). The very short presentation time of the stimulus, which is necessary for a human to extract the emotional content, was investigated by Junghöfer, Bradley, Elbert, and Lang (2001). In their fleeting images paradigm, emotionally arousing and neutral pictures of the international affective picture system (IAPS) were serially presented alternating between high and low arousing content. Remarkably, in early ERPs as well as in fMRI, blocks of rapidly presented arousing pictures elicited increased brain activation in the secondary visual cortex compared to low arousing picture blocks (Junghöfer et al., 2006). However, the stimulus presentation in blocks could lead to a categorization of the picture content and therefore to a change of the context. The long-term memory consolidation of individual stimuli might be inhibited because of the rapid presentation series and masking effects (Potter et al., 2002). According to Junghöfer et al.'s (2001) fleeting images paradigm, EEG provides the opportunity to record early attention processes (Luck et al., 2000) and affective stimulus processing (for review see Olofsson et al., 2008). In particular, the P100, a positive voltage change, which emerges around 100 ms after stimulus onset in the occipital cortex (Hillyard & Münte, 1984), is modulated by attention as attended stimuli elicit larger ERP amplitudes relative to ignored stimuli (Clark & Hillyard, 1996; Rugg, Milner, Lines & Phalp, 1987). Additionally, emotional stimuli like pictures and faces evoked a larger early posterior negativity (EPN) around 240-280 ms after stimulus onset at the occipital and

parietal scalp areas (Mühlberger et al., 2009; Schupp, Junghöfer, Weike & Hamm, 2004a; Schupp et al., 2004b). P100 and EPN can also be modulated by the physical properties of the stimuli (Bradley, Hamby, Löw & Lang, 2007). Later during stimulus processing, the late positive potential (LPP) arises in central and parietal cortex in a time window between 400 and 1000 ms after stimulus onset (Bradley et al., 2007; Kastner, Flohr, Pauli & Wieser, 2016; Schupp et al., 2004a). The LPP is modulated by motivational relevance in that emotionally arousing stimuli elicited a higher LPP amplitude compared to neutral stimuli (Bradley et al., 2007; Schupp, 2000).

In the current study, I combined context conditioning in VR with multiCS conditioning and recorded ERPs as well as subjective ratings of valence, arousal, anxiety, and contingency. To this end, I aimed to develop a paradigm for disentangling the electro-cortical responses to conjunctive and elemental representations of a context in humans. To this purpose, I took sequential screenshots of two virtual offices as if an observer who moved through the rooms took them. During the experiment, these screenshots were subsequently presented like a flip-book, so that participants got the impression to be guided through the rooms. This experimental design gave me the opportunity to pair single screenshots of one context (anxiety context, CTX+) with an US (threat elements) and at the same time other screenshots of the same context not pair with the US (non-threat elements), which investigated elemental representation. On the other hand, the comparison between screenshots of CTX+ and screenshots depicting another context (safety context; CTX-) revealed results on conjunctive context representation. My goal was to disentangle learning mechanisms involving elemental and conjunctive representation by analyzing and comparing the subjective and electro-cortical responses elicited by feature and context stimuli, i.e. screenshots after which the US was presented and screenshots of the room in which the US was presented.

Therefore, I hypothesized conjunctive context representation to be indicated by lower valence and higher arousal, anxiety, and contingency ratings as well as higher electro-cortical activity elicited by all CTX+ compared to CTX- screenshots. Additionally, I hypothesized higher electro-cortical activity elicited by the screenshots specifically paired with an US compared to screenshots of the same context that have never been paired with an US. This would represent elemental representation.

2.2 Material and methods

Katrin Freundorfer wrote her Master Thesis in the context of this study under my supervision.

2.2.1 Participants

Participants were recruited from the internet platform SONA (psywue.sona-systems.com) by the University of Würzburg. In total, 43 participants took part in the experiment. Fifteen participants had to be excluded due to the accumulation of artifacts in the EEG signal (N = 9; see data reduction), incomplete EEG data recording (N = 2), due to quitting the experiment (N = 1), their left-handedness (N = 2) or due to previous experience with context conditioning (N = 1). Subsequently, 28 participants (17 females; mean age = 26.00 years, *SD* = 7.90) were included in the final analyses.

Twenty-four participants were considered contingency aware because they were able to identify the CTX+ after the acquisition. Since contingency awareness might influence cue as well as context conditioning (Andreatta et al., 2015a; Sevenster, Beckers & Kindt, 2014), only these 24 aware participants (17 females) were analyzed. As results did not change after the inclusion of the unaware participants, I report results for all 28

participants. The study was approved by the ethics committee of the Department of Psychology of the University of Würzburg.

2.2.2 Stimulus Material

Conditioned stimuli (CS). The conditioned stimuli comprised 49 screenshots of two neutral virtual offices, respectively. Every 2 seconds, screenshots were recorded while a virtual agent was guided on a pre-recorded path through the offices, observing the contexts in the first-person perspective. All paths began in a corridor in front of the respective office and terminated in the corridor. Twelve screenshots (threat elements) of one context, the anxiety context (CTX+), were selected to be associated with the US in the acquisition phase. Importantly, the selected screenshots ranged from start until the end of the screenshot sequence but did not depict the entrance door in the foreground. Twelve different screenshots with the same selection criteria (non-threat elements) depicting the same context were never associated with the US. Likewise, 12 screenshots of the second office, the safety context (CTX-) were selected as safety elements. Anxiety and safety context, as well as CSs were counterbalanced across participants. In a pilot study (see Genheimer, 2014), 14 participants (8 females, age: M = 24.7, SD = 3.97) scored the screenshots on a 9-point Likert scale regarding valence (1 = unpleasant; 9 = pleasant), arousal (1 = not arousing; 9 = very arousing), and complexity (1 = figure-ground contrast; 9 = scene). Brightness and entropy of the screenshots were assessed with Adobe Photoshop CS4 (version 11.0, Munich, Germany). Separated Analyses of Variance (ANOVAs) were calculated including the within-subject factors context (Room 1, Room 2) and element (Selection 1, Selection 2 demonstrating which of the 2 x 12 chosen screenshots of each room were employed as threat and non-threat elements or as safety elements) for valence, arousal, complexity, brightness, and entropy, respectively.

Statistics showed no differences in all subjective and objective factors between the screenshot selections (all *p*s > .061) suggesting an appropriate set of stimuli for following conditioning study.

Unconditioned stimulus (US). Mildly painful electric stimuli served as unconditioned stimulus and were delivered to the participants' right inner forearm. The electric stimulus was administered in a frequency of 50 Hz and a duration of 200 ms generated by a constant current stimulator (Digitimer DS7A, Digitimer LTD., Welwyn Garden City, UK). In order to guarantee that the stimulus is mildly painful for each participant, I determined the participant's individual pain threshold. Therefore, several electric stimuli were administered and the participants rated their pain verbally on an 11-point Likert scale (0 = no sensation at all, 4 = pain perceptible, 10 = unbearable pain). Starting with 0 mA, I applied stimuli with increasing intensity in steps of 0.5 mA until the participant rated the stimulation with higher than 4. Afterward, I decreased the intensity in steps of 0.5 mA until the participant rated the stimulation lower than 4. The increasing and decreasing series of the stimulation intensity was iterated twice. Afterward, the average of the four threshold intensities, which were scored with 4, was assessed. To assure that the stimulus during the study stayed slightly above pain perception, I added 30% of the stimulation intensity to the calculated average. This electric stimulus was eventually delivered and scored by the participant. The mean intensity was 1.43 mA (*SD* = 0.70), the mean rating score was 6.14 (*SD* = 1.21). I explained that all electric stimuli delivered during the study will be adapted to this pre-determined stimulation intensity. Participants were informed that they can predict the electric stimuli if they pay attention to the experiment.

2.2.3 Measures

Questionnaires. The following questionnaires were completed during the experiment: A demographic questionnaire contained age, gender, education, profession, and handedness. Furthermore, the Anxiety Sensitivity Index (ASI; German Version by Alpers & Pauli, 2001; Reiss, Peterson, Gursky & McNally, 1986) was filled in to assess participants' anxiety and to predict whether the participant will react fearfully. Anxiety sensitivity is referred to as the participants' negative implications of experienced anxiety (Reiss et al., 1986). The questionnaire consists of 16 items (e.g. '*It is important to me not to appear nervous'*) which can be answered on a 5-point Likert scale from 0 (*very few*) to 4 (*very much*). The State-Trait Anxiety Inventory (STAI) assesses participants' current and general anxiety (German Version by Laux, Glanzmann, Schaffner & Spielberger, 1981; Spielberger, Gorsuch & Edward, 1970). The trait part of the STAI consists of 20 items (e.g. '*I am a steady person*'), requesting participants' general anxiety on a 4-point Likert scale (1 = *almost never* until 4 = *almost always*). The state version of this questionnaire also contains 20 items (e.g. '*I feel secure*') and may be answered on a 4-point Likert scale (1 = *not at all* until 4 = *very much so*). The STAI state was filled in twice, i.e. pre and post experiment. Likewise, the Positive Affect Negative Affect Schedule (PANAS; German Version by Krohne, Egloff, Kohmann & Tausch, 1996; Watson, Clark & Tellegen, 1988) was assessed pre and post experiment. The questionnaire acquires the participants' mood on a positive scale (positive affect, PA) as well as on a negative scale (negative affect, NA). It consists of a list of 20 words describing various feelings and emotions (e.g. '*interested*'). Participants are requested to state their momentary feeling on a 5-point Likert scale ranging from 1 (*very slightly or not at all*) until 5 (*extremely*). The Igroup Presence Questionnaire[1] (IPQ; Original Version in German: Schubert, Friedmann & Regenbrecht,

[1] Please note that one participant did not fill in the second page of the IPQ, therefore these values were interpolated on the basis of the grand mean of all other participants.

2001) assessed the participants' feeling of being present in the virtual environment. The IPQ consists of four subscales General, Spatial Presence, experienced Realism, and Involvement. Participants' experience of the feeling of being present in a virtual reality environment was measured retrospectively with 14 items (e.g. '*In the computer-generated world I had a sense of "being there."*'). The 7-point Likert scale reaches from -3 (e.g. *not at all/fully disagree* or the like) to +3 (e.g. *very much/fully agree*).

The analyses of all trait questionnaires revealed unremarkable traits (Table 1). STAI state (Laux et al., 1981; Spielberger et al., 1970) and negative affect (Krohne, Egloff, Kohmann, Tausch, 1996) did not alter over the course of the experiment (STAI: $t(27) = 1.51$, $p = .142$; NA: $t(27) = 0.34$, $p = .733$). The positive affect decreased after the experiment compared to before ($t(27) = 5.22$, $p < .001$) which is similar to previous aversive conditioning experiments (Andreatta et al., 2015b).

Table 1: Overview of the scores on trait questionnaires – Study 1.

Questionnaire	**Mean (*SD*)**
ASI (*SD*)	15.00 (7.17)
STAI trait (*SD*)	38.32 (9.84)
IPQ General Presence (*SD*)	2.96 (1.84)
IPQ Spatial Presence (*SD*)	2.88 (1.42)
IPQ Involvement (*SD*)	2.09 (1.21)
IPQ experienced Realism (*SD*)	2.06 (1.20)

Subjective Ratings. While the picture of one virtual context was presented for 1 s, participants gave their ratings by moving a red arrow below the Likert scale with the computer keyboard. In this way, both contexts (CTX+ and CTX-) were rated on valence

(*How positive or negative was this room for you?*) from 0 = *very negative* to 50 = *neutral* to 100 = *very positive*, arousal (*How arousing was this room for you?*) from 0 = *no arousal* to 100 = *very high arousal*, anxiety (*How anxious did you feel in this room?*) from 0 = *no anxiety* until 100 = very high anxiety, and contingency (*How likely did you receive an electric shock in this room?*) from 0% (*very unlikely*) to 100% (*very likely*). Before each rating, a picture of the respective context was shown for 2 s.

Additionally, we assessed contingency ratings for the three categories of screenshots (threat, non-threat and safety elements). The following question was presented on the computer screen: *Have you received an electric stimulus at this place of the room earlier?* Participants selected their answer on an 11-point Likert scale from 0 (*surely no US*) until 10 (*surely US*) by pressing the respective buttons on the keyboard.

Electroencephalography (EEG). The electroencephalogram was recorded by 28 Ag-AgCl electrodes according to the international 10-20 system (Fp1, Fp2, F7, F3, Fz, F4, F8, FC5, FC1, FC2, FC6, T7, C3, Cz, C4, T8, CP5, CP1, CP2, CP6, P7, P3, Pz, P4, P8, O1, Oz, O2). The electrodes were attached to a cap and placed on the scalp (Acticap, Brain Products GmbH, Munich, Germany). Additionally, four electrodes were attached to participants' faces to control for eye movements. Horizontal eye-movements were controlled by two electrodes placed on the left and right canthi, vertical eye-movements were controlled by two electrodes centrally above and beneath the left eye. The electrode Fz was used as ground electrode, Cz served as online reference. An electrolyte gel (EASYCAP GmbH, Herrsching, Germany) was used to keep the impedances below 5 kΩ. This was ensured using Acticap control software (version 1.2.2.0, Brain Products GmbH, Munich, Germany). The sampling rate was 1000 Hz. An online Notch filter of 50 Hz was applied. Data were recorded with Vision Recorder software (version 1.20, Brain Products GmbH, Munich, Germany).

2.2.4 Procedure and Design

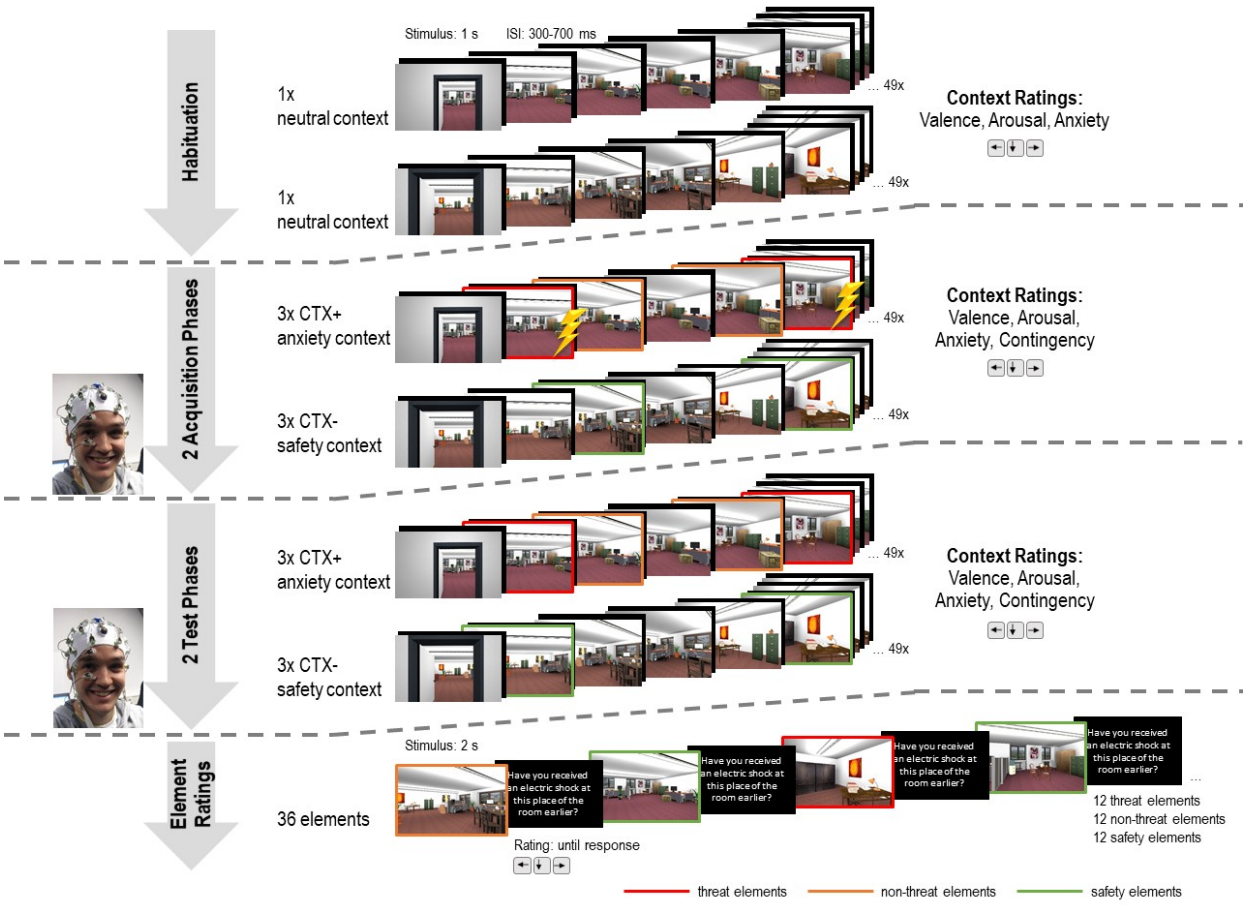

Figure 1: Experimental procedure of Study 1.

During Habituation, the two virtual rooms were shown once, i.e. the 49 screenshots of each office were shown in subsequent order for 1 s each including an Inter-Stimulus-Interval (ISI) of 300-700 ms. During each Acquisition phase, both contexts were shown three times similar to Habituation, but, 12 screenshots (threat elements, red frame) of the anxiety context (CTX+) were followed by mildly painful electric stimuli (USs). The same number of comparable CTX+ screenshots (non-threat elements, orange frame) and screenshots depicting the safety context (CTX-; safety elements, green frame) represented control stimuli. Both Test phases were identical with the Acquisition phases, however without US delivery. Ratings were retrieved after Habituation, both Acquisition and both test phases. After this procedure, the 12 threat, the 12 non-threat, and the 12 safety screenshots were shown once for 2 s in randomized order (element ratings) and participants reported the probability of having received an US at the offset of this particular screenshot during the study.

The experiment was controlled with *Presentation* (version 15.1, Neurobehavioral Systems, Inc.). All instructions, pictures, and rating scales were presented on a Powerwall, which is a big screen of 3.22 m in length and 2 m in height allowing to depict furniture and contextual stimuli in about full-size. During the study, participants sat about 2 m in front

of the Powerwall. In the beginning, participants read the experimental instructions and gave their informed consent. Additionally, an information sheet containing the rating scales that were used during the experiment was provided. The experimenter responsed to potentially arising questions. Then, questionnaires about demographical data, ASI (Alpers & Pauli, 2001; Reiss et al., 1986), STAI trait and state (Laux et al., 1981; Spielberger et al., 1970), as well as PANAS (Krohne et al., 1996; Watson et al., 1988) were filled in. Afterward, the experimenter applied the EEG cap, the electrodes for delivering aversive stimuli, and the determination of the pain threshold was explained and completed.

The full study contained a habituation phase, two acquisition and two test phases, and post-experimental contingency ratings for elements (Figure 1). During habituation, participants saw 49 sequential screenshots of one context followed by 49 screenshots of the other context. Each screenshot was displayed for 1 s, the Inter-Stimulus-Interval (ISI) with a black screen lasted between 300 – 700 ms. A context run refers to the series of all 49 subsequent screenshots of one context. After habituation, participants scored valence, arousal, and anxiety of both rooms. During acquisition phase 1 (A1), participants observed the blocks of 49 screenshots of both contexts again, three times for each context (order: CTX+, CTX-, CTX+, CTX-, CTX-, CTX+). In one room (anxiety context, CTX+), 12 screenshots (threat elements) were once presented with the US applied at the screenshot's offset. Therefore, in sum 12 USs were administered during A1, three to five USs per context run. A screenshot was described as a threat element only when it has been paired with the US before. Twelve comparable screenshots of the anxiety context represented non-threat elements, and 12 comparable screenshots of the CTX- served as the safety elements. A1 ended with the assessment of valence, arousal, anxiety, and contingency for each room. The second acquisition phase (A2) contained the same

procedure, but with an altered sequence of context runs (CTX-, CTX+, CTX+, CTX-, CTX+, CTX-). As in A1, the same 12 threat elements of CTX+ were once paired with the US, the same 12 non-threat elements of the anxiety context and the same 12 safety elements of the safety context were included in the analyses. The two test phases (T1 and T2) were identical and similar to the acquisition phases, but without US administration and with new context run sequences (T1: CTX-, CTX+, CTX-, CTX+, CTX+, CTX-; T2: CTX+, CTX-, CTX-, CTX+, CTX-, CTX+). Lastly, the contingency ratings at the end of the study were conducted by presenting all 12 threat, 12 non-threat, and 12 safety elements for 2 s in random order. Participants reported the probability that the US was delivered after exactly this screenshot of this room earlier in the study.

I counterbalanced CTX+ and CTX-, as well as threat, non-threat, and safety elements across participants. The study was finished with the completion of the PANAS (Krohne et al., 1996; Watson et al., 1988), STAI state (Laux et al., 1981; Spielberger et al., 1970) and IPQ (Schubert et al., 2001).

2.2.5 Data Recordings and Data Reduction

Subjective Ratings. The ratings of all subjective data were saved in a Microsoft Excel-sheet and imported into IBM SPSS Statistics 23 (IBM Corporation, Armonk, New York, USA). There, all analyses were calculated.

Electroencephalography. In this experiment, I analyzed the amplitudes of the ERP components P100, EPN, and early and late LPP. For data preprocessing I used BrainVision Analyzer 2.1 software (Brain Products Inc., Munich, Germany). First, a new reference was calculated by using all electrodes except the electrodes around the eyes. A bandpass filter from 0.1 Hz to 35 Hz was applied as well as a Notch filter of 50 Hz. Ocular correction was performed according to Gratton, Coles, and Donchin (1983) by a blink detection algorithm

as implemented in BrainVision Analyzer. Then, temporal segments from 100 ms before until 1000 ms after each screenshot onset were extracted, baseline corrected, and proved for artifacts (rejection criteria allowed a maximal voltage step of 50 μV, a minimal amplitude of -100 μV and a maximal amplitude of 100 μV; see Wieser, Pauli, Reicherts & Mühlberger, 2010). Afterward, segments were averaged across each experimental condition respectively.

A peak detection between 80 and 160 ms after stimulus onset was performed for the analysis of the P100. The values of these peak amplitudes of the occipital electrodes O1 and O2 were exported, transferred into IBM SPSS Statistics 23 (IBM Corporation, Armonk, New York, USA), and averaged for further analyses. Furthermore, I exported the mean amplitudes 180-240 ms after stimulus onset of the electrode cluster P7, P8, O1, and O2 for the EPN, and the mean amplitudes 400-700 ms as well as 700-1000 ms after stimulus onset of Pz for early and late LPP, respectively.

2.2.6 Statistical Analyses

For the calculation of all analyses I used IBM SPSS Statistics 23 (IBM Corporation, Armonk, New York, USA).

Ratings. I examined subjective rating data of the contexts (valence, arousal, anxiety, and contingency) with paired samples *t*-tests for the habituation and with repeated measures 2 x 2 ANOVAs containing the within-subject factors context (CTX+, CTX-) and time (A1, A2 or T1, T2) for the acquisition and test phases. Post-experimental contingency ratings of the elements were examined by calculating an ANOVA with the within-subject factor element containing 3 levels (threat, non-threat, safety). Importantly, data of only 16 participants were included in this analysis. For further participants, the experiment terminated after the test phase, no rating data of the elements were collected.

Physiology. EEG findings were reported according to Picton et al. (2000). I separately analyzed each ERP component in the habituation phase with ANOVAs with the within-subject factor element (threat, non-threat, safety); for the EPN with the additional factor hemisphere (left, right). The acquisition and the test phases were examined for P1 as well as early and late LPP with 2 x 3 ANOVAs with the within-subject factors phase (A1, A2 or T1, T2) and element (threat, non-threat, safety) and for the EPN with a 2 x 2 x 3 ANOVA containing the extra factor hemisphere (left, right). Importantly, in A1 a screenshot became a threat element not until it has been paired with the US in the previous context run. To this end, A1 contained 11 threat screenshots, which were compared to 36 non-threat and 36 safety elements for each participant. In A2, all 12 threat elements had been paired with the US previously, therefore A2 comprised 36 threat elements.

All significant interactions and main effects were followed-up by post-hoc *t*-tests. In case of violating the sphericity assumption, Greenhouse-Geisser corrections were applied and Greenhouse-Geisser Epsilon (*GG-*ε) reported. The α-level was set at 0.05. For effect sizes, Partial eta square (η_p^2) is reported.

Exploratory Analyses. ASI and STAI trait scores were correlated with EPN using Pearson's correlation. STAI state and PANAS PA, as well as NA were compared pre and post experiment with paired *t*-tests. P-values and Pearson's correlation coefficient *r* were reported.

2.3 Results

2.3.1 Ratings for contexts

Ratings after the habituation phase revealed that participants rated both offices not significally different regarding valence, arousal and anxiety (all *p*s > .056, see Figure 2), thus inferring that the virtual rooms were suitable for the following study design.

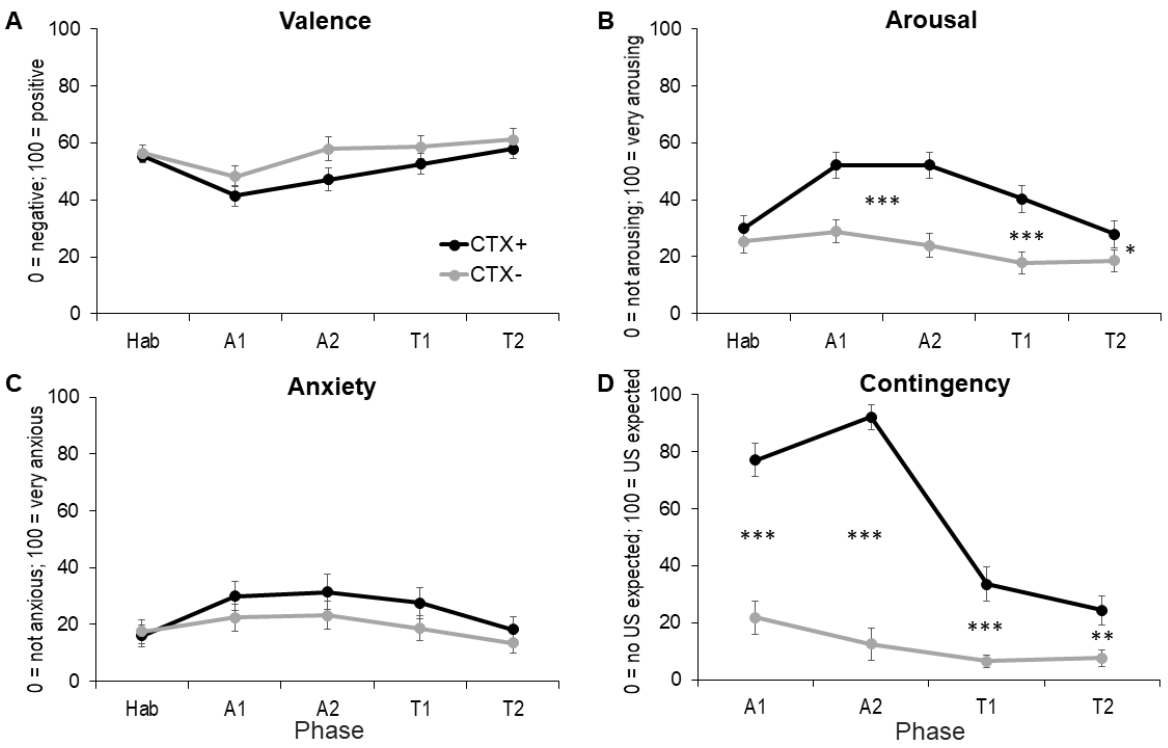

Figure 2: Ratings of the contexts in Study 1.

Ratings of valence (A), arousal (B), anxiety (C) and contingency (D) for anxiety context (CTX+, black lines) and safety context (CTX-, gray lines) with standard errors (SE). Valence, arousal, and anxiety ratings were reported after habituation (Hab). Valence, arousal, anxiety, and contingency ratings were assessed after Acquisition 1 (A1) and 2 (A2) as well as after Test 1 (T1) and 2 (T2). *** $p < .001$; ** $p < .01$; * $p < .05$

Valence. During acquisition, the valence ratings only showed a significant main effect of phase ($F(1,27) = 7.52$, $p = .011$, $\eta_p^2 = .218$) demonstrated by lower pleasantness after A1 than after A2, which could either reveal negative valence caused by aversive anxiety conditioning or showing the negative mood or reflecting habituation to the experimental setting. Neither the effect of context nor the interaction of Phase x Context became significant (all *p*s > .171).

For the test phase, I detected similar effects: A main effect of phase revealed lower valence in T1 compared to T2 ($F(1,27) = 11.46$, $p = .002$, $\eta_p^2 = .298$) showing a further increase in pleasant valence, probably due to the absence of painful stimulation after

acquisition. However, the effect of context and the interaction of Phase x Context did not reach significance level (all *p*s > .301; Figure 2A).

Arousal. Successful anxiety acquisition was demonstrated by a main effect of context ($F(1,27) = 28.92$, $p < .001$, $\eta_p^2 = .517$) with lower arousal ratings for CTX- versus CTX+. The main effect of phase and the interaction of Phase x Context did not turn out significant (all *p*s > .440). Analyses of the test phase indicated slow extinction learning as revealed by a significant main effects of phase ($F(1,27) = 5.30$, $p = .029$, $\eta_p^2 = .164$), context ($F(1,27) = 17.76$, $p < .001$, $\eta_p^2 = .397$) and a significant interaction of Phase x Context ($F(1,27) = 15.70$, $p < .001$, $\eta_p^2 = .368$). Post-hoc *t*-tests demonstrated lower arousal ratings for CTX- versus CTX+ after T1 ($t(27) = 5.32$, $p < .001$) and after T2 ($t(27) = 2.31$, $p = .029$; Figure 2B).

Anxiety. On a descriptive level, the acquisition phase elicited increased anxiety in CTX+ compared to CTX- (see Figure 2C), however, the statistical analyses indicated neither a significant effect of phase, nor context, nor of Phase x Context (all *p*s > .257). The ANOVA for the test phase demonstrated only a main effect of phase ($F(1,27) = 11.27$, $p = .002$, $\eta_p^2 = .295$) illustrating higher anxiety after T1 than after T2. The interaction Phase x Context was not significant ($F(1,27) = 2.02$, $p = .167$, $\eta_p^2 = .069$) despite anxiety ratings looked descriptively higher for CTX+ than CTX- after T1, but on a similar level after T2.

Contingency of contexts and US. A significant main effect of context ($F(1,27) = 95.75$, $p < .001$, $\eta_p^2 = .780$) and a significant interaction of Phase x Context ($F(1,27) = 7.42$, $p = .011$, $\eta_p^2 = .216$) during acquisition demonstrated successful anxiety learning. Post-hoc *t*-tests for the interaction effect indicated significantly higher contingency ratings for CTX+ than

CTX- after A1 ($t(27) = 6.21$, $p < .001$) and after A2 ($t(27) = 10.70$, $p < .001$). The analysis of the test phase revealed a significant interaction of Phase x Context as well ($F(1,27) = 4.25$, $p = .049$, $\eta_p^2 = .136$) demonstrating higher contingency ratings for CTX+ than CTX- after T1 ($t(27) = 4.50$, $p < .001$) and after T2 ($t(27) = 3.56$, $p = .001$). These findings illustrate uncompleted extinction during the test phases (Figure 2D).

2.3.2 Contingency ratings of elements at the end of the experiment

The main effect of element ($F(2,30) = 40.66$, $GG\text{-}\varepsilon = .593$, $p < .001$, $\eta_p^2 = .731$) suggests elemental representation as the post-hoc *t*-tests revealed higher contingency ratings for threat versus non-threat elements ($t(15) = 2.44$, $p = .028$) or versus safety ($t(15) = 6.48$, $p < .001$) elements. The same is true for non-threat compared versus safety elements ($t(15) = 6.77$, $p < .001$). These findings, on the one hand, show conjunctive representation as participants reported higher US contingencies for all screenshot elements illustrating the anxiety context (both threat and non-threat elements) compared to screenshot

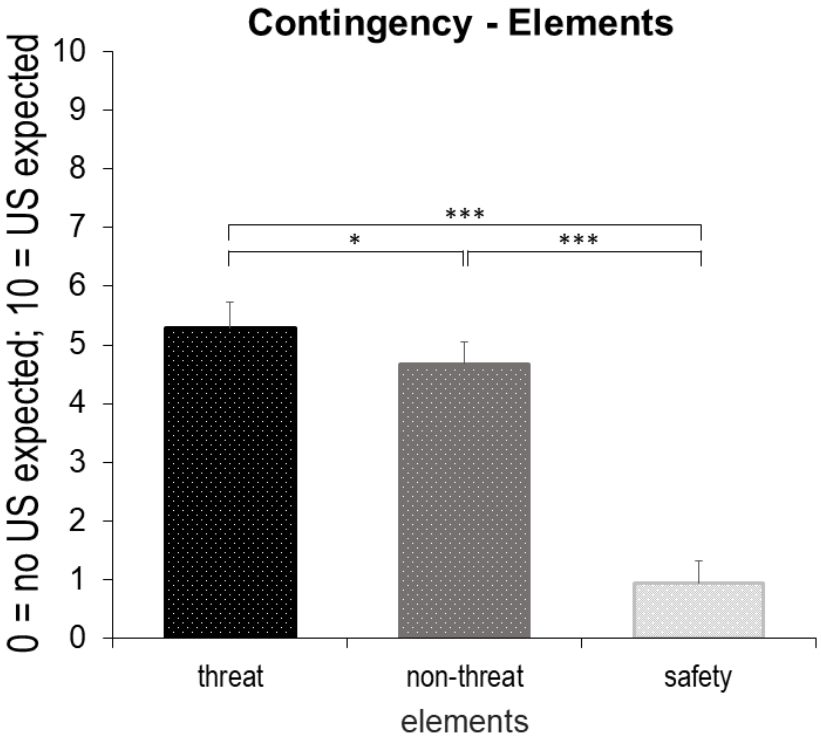

Figure 3: Contingency ratings of elements in Study 1.

At the end of the experiment, contingency ratings for the 12 preselected screenshots of each condition illustrating elemental representation are displayed. Mean US contingency ratings and standard errors (SE) are shown for screenshots depicting CTX+ either paired with an US during acquisition (threat elements, black bars) or not paired with an US during acquisition (non-threat elements, dark gray bars) or matched control screenshots displaying the safety context (safety elements, light gray bars). *** $p < .001$; * $p < .05$

elements depicting the safety context (safety elements). Importantly, results, on the other hand, provide evidence for elemental representation because contingency ratings between threat and non-threat elements differed. This suggests a very precise differentiation between reinforced and non-reinforced CTX+ elements. Please note that these results also suggest weak extinction as these ratings were assessed after both test phases in which no US was administered (Figure 3).

2.3.3 ERP responses to contextual elements

The analyses of the ERP components P100, EPN, early LPP, and late LPP during the habituation phase provided no significant effects of element (all *p*s > .123) demonstrating a proper baseline setting for further calculations.

P100. The analyses of acquisition indicated a main effect of phase ($F(1,27) = 4.20$, $p = .050$, $\eta_p^2 = .135$) revealing higher P100 amplitudes during A1 versus A2. The main effect of element ($F(2,54) = 4.43$, $p = .017$, $\eta_p^2 = .141$; Figure 4A) deduced from increased P100 signal for threat versus safety elements ($t(27) = 2.40$, $p = .024$) and for non-threat versus safety elements ($t(27) = 3.18$, $p = .004$). No difference was found between amplitudes elicited by threat and non-threat elements ($t(27) = 0.13$, $p = .899$). The interaction of Phase x Element was not significant ($F(2,54) = 0.26$, $GG\text{-}\varepsilon = .733$, $p = .704$, $\eta_p^2 = .009$). This illustrates differential processing of screenshots depicting the anxiety context compared to screenshots showing the safety context, suggesting conjunctive context representation. The analysis of the test phase revealed neither an effect of phase nor of element nor the interaction Phase x Element (all *p*s > .076), suggesting successful extinction.

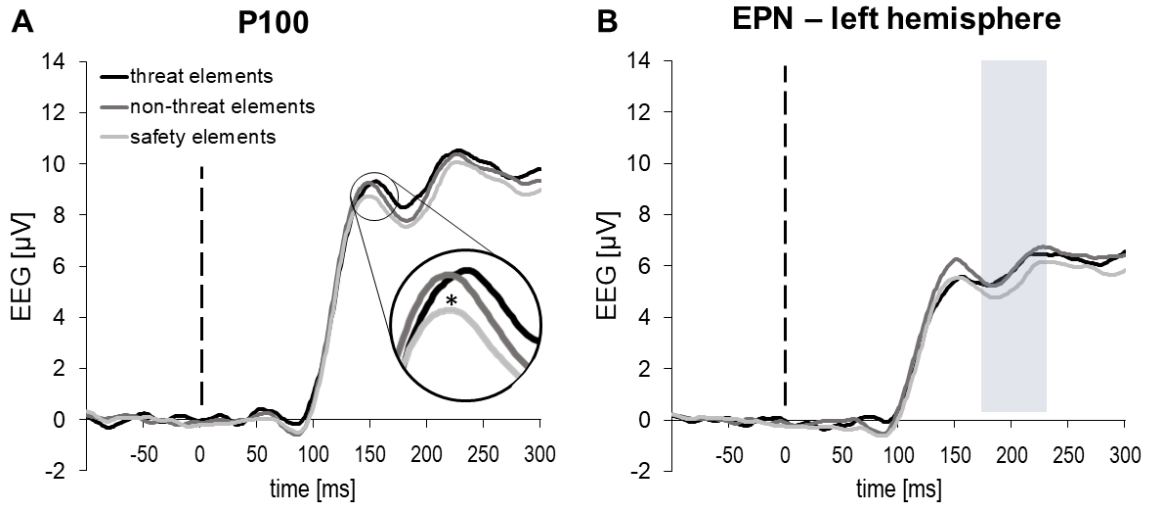

Figure 4: Mean P100 and EPN amplitudes during acquisition in Study 1.

The figure illustrates (A) mean P100 amplitudes for the electrodes O1, Oz, O2 and (B) mean EPN amplitudes for the electrodes P7 and O1 of the left hemisphere elicited by threat (black line), non-threat (dark gray line), and safety (light gray line) elements.

Early posterior negativity (EPN). The acquisition analysis revealed an interaction of Element x Hemisphere ($F(2,54) = 5.63$, $GG\text{-}\varepsilon = .557$, $p = .021$, $\eta_p^2 = .172$). Post-hoc *t*-tests for elements in the left hemisphere indicated lower EPN amplitudes for safety elements versus threat ($t(27) = 2.18$, $p = .038$) and non-threat elements ($t(27) = 2.37$, $p = .025$), while threat and non-threat elements elicited comparable EPN amplitudes ($t(27) = 0.51$, $p = .612$; see Figure 4B). In contrast, post-hoc tests for the right hemisphere revealed similar amplitudes elicited by all different elements (all *p*s > .360). These results demonstrate an electro-cortical differentiation between threat and non-threat CTX+ compared to CTX- elements in the left hemisphere, suggesting conjunctive context representation. Neither main effects of phase and element, nor the interaction thereof became significant during the test phase (all *p*s > .077), indicating successful extinction learning.

Early component of the late positive potential (LPP; 400-700 ms). The analyses of the early component of the late positive potential revealed neither an effect of element ($F(2,54) = 0.10$, $GG\text{-}\varepsilon = .770$, $p = .851$, $\eta_p^2 = .004$), nor of phase ($F(1,27) = 0.38$, $p = .542$, $\eta_p^2 = .014$), nor of the interaction of both ($F(2,54) = 0.68$, $p = .510$, $\eta_p^2 = .025$) during acquisition. This speaks for a lack in learning of the motivational relevance of distinct screenshots. Without learning during acquisition, no effects could be found during test, neither main effect of phase, nor element, nor an interaction of both (all *p*s > .095).

Late component of the late positive potential (LPP; 700-1000 ms). The analyses of the late LPP revealed no effects during acquisition, neither of element, nor of phase, nor of the interaction of both (all *p*s > .677). Similarly, the ANOVA for the test phase revealed neither effects of phase, element, nor of the interaction of Phase x Element (all *p*s > .177).

2.3.4 Exploratory analyses

Participants' anxiety sensitivity and trait anxiety might affect fear learning and especially on the ERP component EPN, which depicts the electro-cortical processing of the emotional value of a stimulus. I, therefore, correlated the EPN amplitudes at A1 and A2, with participants' ASI and the STAI scores (see Table 2). The negative correlations show the higher either the ASI or the STAI trait scores are the more negative are the EPN amplitudes. This is an indicator of emotional stimulus content. Anxiety sensitivity did not significantly correlate with EPN amplitudes (all *p*s > .314; see Table 2). Interestingly, in A1 participants who scored high on trait anxiety had lower EPN amplitudes for screenshots depicting CTX+, i.e. threat (p = .010; r = -.480) and non-threat (p = .037; r = -.395) elements, but not for elements depicting the safety context, i.e. safety elements (p = .114; r = -.306; see Table 2). The effect disappeared in A2 (all *p*s > .114).

Table 2: Correlation analyses of EPN and ASI/STAI trait in Study 1.

EPN amplitudes of threat, non-threat and safety elements during acquisition 1 and 2 were correlated with ASI or STAI trait, respectively.

EPN/ASI	**Threat**	**Non-threat**	**Safety elements**
A1	p = .330; r = -.191	p = .813; r = -.047	p = .812; r = -.047
A2	p = .315; r = -.197	p = .604; r = -.103	p = .345; r = -.185
EPN/STAI trait			
A1	***p* = .010; *r* = -.480**	***p* = .037; *r* = -.395**	p = .114; r = -.306
A2	p = .380; r = -.173	p = .242; r = -.229	p = .115; r = -.304

2.4 Discussion

The goal of the current study was the investigation of the conjunctive and the elemental representation of a context in one experimental paradigm. Therefore, I used sequentially taken screenshots of two virtual offices that simulated a first-person perspective movement through the contexts. Such a sequential presentation of the screenshots, on the one hand, elicits ERPs by the onset of each screenshot and on the other hand allows subjective reports on single elements, i.e. single screenshots, as well as on both conjunctive contexts. With this method, I could disentangle the dual representations of a context. Regarding the recorded ERP responses as well as the verbal ratings, our current study found the first evidence for the dual context representation in humans within one paradigm (Nadel & Willner, 1980; Rudy, 2009; Rudy et al., 2004). To this end, larger P100 and EPN amplitudes and higher arousal and contingency ratings for threat as well as non-threat elements, both depicting the threat context, compared to safety elements illustrating the safety context, revealed conjunctive representation of the threat context. In parallel, participants were indeed able to discriminate single elements of the anxiety context by reporting higher contingency ratings for threat elements, i.e. screenshots that were followed by the US, compared to non-threat elements of the same context. Therefore, evidence for conjunctive representation on the electrophysiological and subjective level as well as for elemental representation of CTX+ on the subjective level indicated the reliable investigation of the dual representation of a context using the newly developed flip-book paradigm.

Our results of enhanced electro-cortical responses and verbal reports to all elements depicting the anxiety context compared to elements illustrating the safety context confirm and extend previous context conditioning studies (e.g., Glotzbach-Schoon et al., 2013a). Higher arousal and US expectancy ratings in CTX+ compared to CTX- indicated consistent

context conditioning which corroborates the comparability of the flip-book paradigm with previous context conditioning studies (Andreatta et al., 2015b; Baas et al., 2004). Additionally, previous findings were extended as indicators for conjunctive context representation were revealed with ERPs for the first time in the current study. In particular, all screenshots of the anxiety context, independent from threat-related or threat unrelated elements, triggered enhanced P100 and EPN amplitudes compared to screenshots of CTX-. However, for the LPP I did not find such differences. In sum, conjunctive representation of a context was emphasized only in early ERP components and independent of the de facto association with the threat.

More precisely, early attentional processes have been investigated in studies presenting pictures of checkerboards or faces on a computer screen revealing higher P100 amplitudes for attended vs. unattended pictures (Clark & Hillyard, 1996; Wieser et al., 2010). The physical properties of the pictures were also shown to modulate the P100 amplitude. Here, we carefully controlled stimuli for the picture size, complexity, and illumination (Genheimer, 2014) and therefore assume that any modulations of the P100 amplitude originate from our experimental manipulations rather than from physical properties. To this end, higher arousal for CTX+ could have resulted in higher overall attention for the anxiety compared to the safety context. Therefore, CTX+ elicited larger P100 amplitudes indicating conjunctive representation of the context. In the subsequent test phase, no USs were delivered, and participants' arousal level decreased in the anxiety context which resulted in similar levels of attention and similar P100 amplitudes for screenshots of both contexts. Alternatively, context-dependent learning is facilitated by directing the attention towards the context (Rosas, Aguilera, Álvarez & Abad, 2006). Besides, both ratings and US delivery in the anxiety context might have enhanced the saliency of the anxiety context, which could have resulted in enhanced P100 and EPN

amplitudes. Current contingency ratings further support such learning facilitation. Thus, at the end of each phase of the experiment, participants differentiated US expectancy ratings between CTX+ and CTX-. Moreover, both threat and non-threat elements were considered as associated with the US, likely due to generalization processes. The screenshots were taken every 2 seconds from the first-person perspective of the virtual agent moving through the context. As a result, adjacent screenshots depicted similar views of the context and its elements from different angles. Therefore, one feature, e.g. a desk, which was present in a threat screenshot, might also have been visible in a subsequent non-threat screenshot and again elicited a fear response in the ERP. Such similarities in the screenshots might have facilitated the conjunctive context rather than the elemental representation. Those single objects, which were present in several screenshots and sometimes associated with an US, might have increased the general attention in this context, leading to increased P100 amplitudes for all CTX+ screenshots compared to CTX- screenshots. However, even though threat and non-threat elements shared many features because they depicted the same context, they are distinct from safety elements of the safety context, which may have dampened differential ERP responses within the anxiety context.

Likewise, the emotional content of a stimulus strongly modulates EPN amplitudes (Schupp et al., 2004a). In the present study, elements depicting CTX+ compared to CTX- might, in general, elicit higher emotional relevance due to their similarity and generalization effects, which resulted in higher EPN amplitudes. Schupp et al. (2004a) reported that unpleasant compared to pleasant pictures from the IAPS elicited more positive EPN amplitudes. In comparison, we also found more positive EPN amplitudes for all CTX+ compared to CTX- screenshots, independent of their relation to the threat. Therefore, our data prove electrophysiological evidence for successful context

conditioning and subsequently for conjunctive context representation. Interestingly, high trait anxious individuals tended to associate a context with an aversive event (US) faster. Those participants generalized their learned threat element – US associations to any element depicting CTX+. This is in line with a previous context conditioning study, which found faster anxiety conditioning in high compared to low trait anxious individuals in terms of startle responses (Glotzbach-Schoon et al., 2013c). I applied the US to the right forearm of only right-handed participants. Therefore, I speculate that the location of the stimulus presentation might be responsible for the detection of the effect in the left hemisphere. In line with this, pronounced stimulus processing in the contralateral hemisphere of the location of the electric stimulus was already described in a previous study (Andreatta et al., 2015a).

EPN amplitude and amygdala as well as anterior cingulate cortex (ACC) activity correlated negatively in a combined EEG and fMRI study (Sabatinelli, Keil, Frank & Lang, 2013). Therefore, I suggest increased amygdala activity in the current experiment when the threat or non-threat elements were presented. Stout et al. (2018) investigated conjunctive representation by using pictures of contexts with the same elements but different arrangements and reported hippocampus but not amygdala activity. Associating an aversive event with the arrangement of elements only required conjunctive context learning (Stout et al., 2018). In contrast, the current study investigated elemental and conjunctive representations at the same time and therefore required amygdala as well as hippocampal activity. Similarly, ongoing amygdala and hippocampal activity during the presentation of a threat context have previously been found (Andreatta et al., 2015a; Rudy, 2009). Moreover, the tight connection between the amygdala and hippocampus during threat perception has been emphasized (LeDoux & Pine, 2016). To this end, our interpretation of the data seems consistent but further confirmation is highly warranted.

Against my hypothesis, neither the elemental stimuli nor the contextual comparisons modulated LPP amplitudes in my data set. Motivational salience has been shown to modulate LPP amplitudes, reflecting motivational classification of the respective stimulus (Bradley et al., 2007; Kastner et al., 2016; Schupp et al., 2004a). One reason for similar LPP amplitudes in my study might be a comparable motivational salience of both contexts, whereby CTX+ elicited avoidance whereas CTX- elicited approach behavior (Bradley et al., 2001). An additional neutral context in future studies could detect such motivationally induced effects.

In contrast to previous multiCS conditioning studies, I found differentiated contingency ratings between threat and non-threat elements of the anxiety context but not in ERP results. Original multiCS conditioning studies contain a set of stimuli of one category (e.g. faces), which are sequentially presented in random order with some of these stimuli being paired with a US and others not (Steinberg et al., 2013). Despite differential electro-cortical responses to threat-related vs. threat unrelated stimuli, such studies did not find explicit CS-US contingency ratings for threat-related stimuli (Steinberg et al., 2013). However, study paradigms differed: During acquisition in multiCS conditioning, similar but unrelated stimuli are presented in random order, whereas in the flip-book paradigm, contextually linked stimuli are presented in a sequentially fixed order. Therefore, the timing when an US was applied additionally contributed to the recent context conditioning study (Maren et al., 2013). The prediction of an upcoming threatening event, i.e. threat element, is only possible in the flip-book paradigm due to the temporal sequence of the screenshots. Subsequently, the attention of the participants might have been focused on details of the displayed element, expecting the associated US and memorizing these screenshots on an explicit level. During the evaluation of contingency ratings, the elements were presented in random order, but the associative learning has

already been performed during acquisition. An alternative explanation might be the figure-ground phenomenon (Rudy, 2009). Here, elements that were part of the background context but were associated with an US predictive meaning stepped into the foreground and were preferentially processed and memorized (Rudy, 2009). However, both explanations admittedly presume differential processing of threat and non-threat elements also on the electro-cortical level which would lead to differential ERP amplitudes. Likewise, I suggest methodological differences as speculative reasons why I did not find differential ERP amplitudes. In the flip-book paradigm, screenshots belonging to the anxiety and the safety context are very distinct but could have been generalized due to their similar features as argued above. In multiCS conditioning studies, no second context is available which may increase the electro-cortical differentiation between stimuli presented in only one context. Additionally, the relatively limited number of averaged trials in the flip-book paradigm and the therefore large noise compared to the more robust multiCS conditioning trials might be another reason for similar ERP amplitudes elicited by threat and non-threat elements. These speculations have to be considered systematically in future studies.

Moreover, during our initial habituation phase, in which participants were allowed to explore the context and develop an entire spatial map of both contexts, I emphasized conjunctive representation even before conditioning started. In fact, the hippocampus-dependent pre-exposure facilitation effect has been demonstrated in animals (Fanselow, 1990, 2010). Particularly, when such a conjunctive representation already exists before contextual learning, only a small component of this context might activate the entire context representation by pattern completion (O'Reilly & Rudy, 2001; Rudy, 2009; Rudy et al., 2004; Rudy & O'Reilly, 2001). In contrast, single elements might be sufficient to distinguish contexts by pattern separation (O'Reilly & Rudy, 2001; Rudy, 2009; Rudy et

al., 2004; Rudy & O'Reilly, 2001). For future flip-book studies, I suggest either skip the habituation phase or present all screenshots in random sequence to minimize conjunctive representations before conditioning and therefore to increase the chances of finding elemental representations in electro-cortical responses.

At last, the time point when the contingency ratings for the elements were assessed has to be discussed. Namely, I collected those ratings at the end of the experiment after the test phase, i.e. after extinction processes took place. Differential contingency ratings for threat and non-threat as well as for safety elements indicated very slow extinction. Supportively, also the contingency ratings of the contexts that were assessed after the test phase revealed differential ratings and slow extinction on explicit level. Thus, the tight connection between conjunctive and elemental representation is highly conceivable. Additionally, despite the random presentation order of the single elements, participants were able to accurately assign higher contingency ratings to threat compared to non-threat and safety elements, which emphasizes the important role of elemental representation within the dual context representation model.

The flipbook paradigm allows insights into ongoing stimulus processing by continuous ERP recording of the subsequent contextual stimuli. At the same time, it allows the disentanglement of the whole context through the presentation of single screenshots that are related or unrelated to the US. However, a screenshot of a context still contains conjunctive information rather than pure information of one single element. Therefore, one could argue for the high importance of conjunctive rather than elemental representation in the flip-book paradigm. This leads to the question of what can be defined as an element of a context. A screenshot itself indeed might be more than one single contextual feature and therefore always contains conjunctive information per se. To this end, future studies could present single virtual objects rather than screenshots, in

order to even better disentangle both kinds of context representation. Nevertheless, the sequential screenshot representation discloses very early in each context run whether CTX+ or CTX- is presented. For participants, this information provides an adapted state of arousal as well as focused attention towards the threat context which is reflected in ERP results by conjunctive representation. The randomization of the screenshots in the test phase might disentangle prolonged arousal effects in future studies.

To summarize, in this study I directly compared the conjunctive and elemental representation of a context on the electro-cortical as well as subjective level for the first time. Evidence suggested that conditioned contextual anxiety in humans can be attributed to both conjunctive and elemental representation. As such, conjunctive representation was supported by enhanced P100 and EPN amplitudes as well as increased arousal and contingency ratings for threat and non-threat elements depicting CTX+ compared to safety elements illustrating CTX-. Elemental representation was assumed by enhanced contingency ratings for threat vs. non-threat elements both depicting the anxiety context. As a conclusion, the well-controlled flip-book paradigm considers both elemental and conjunctive context representation at the same time. Therefore, this study design as well as current results emphasize the suitability for further research investigating the dual context representation on the electro-cortical and subjective levels. Requirements, dispositions and individual differences causing conjunctive or elemental context representations are possible future investigations in which the flip-book paradigm might be used. Additionally, investigations of the mechanism of contextual representations in PTSD patients or patients with impaired hippocampal functioning might also profit from the flip-book paradigm.

3 Study 2: tVNS and Anxiety

This study has been published in *Scientific Reports* (Genheimer, Andreatta, Asan, Pauli, 2017).

3.1 Introduction

The feeling of anxiety or sustained fear, a diffuse state of anticipation of a possible threat, is essential for an organism's survival (Marks, 1987). Anxiety patients indicate inappropriate responses to threats and impaired fear extinction (Duits et al., 2015; VanElzakker et al., 2014). Therefore, the investigation of associative threat learning by anxiety conditioning paradigms in both rodents (Rudy et al., 2004) and humans (Alvarez et al., 2008; Andreatta et al., 2015a) is essential to understand the underlying mechanisms to draw conclusions on most effective therapy. In such anxiety conditioning paradigms, subjects are exposed to contexts, as defined by Maren et al. (2013), multisensory, diffuse and continuously present circumstances around an event. These contexts may be a cage for rodents (Myers & Gluck, 1994) or a long-lasting color of a computer screen, a picture in the background or a real or a virtual room (Baas et al., 2004; LaBar & Phelps, 2005; Lonsdorf et al., 2014b; Pohlack et al., 2012; Tröger et al., 2012). Importantly, during anxiety acquisition, the anxiety context (CTX+) predicts the aversive, unconditioned stimulus (US) and elicits the diffuse state of anxiety. Such anxiety responses are reflected via freezing behavior (Bolles & Collier, 1976) or startle potentiation (Waters et al., 2014). An anxiety memory trace is created (Bouton, 2002; Quirk & Mueller, 2008). A second context is never paired with an US and becomes the safety context (CTX-). In a subsequent

extinction phase, subjects are exposed to both contexts repetitively, but no US is administered anymore. Hence, an inhibitory extinction memory trace competing with the anxiety memory is formed (Bouton, 2002; Milad & Quirk, 2012; Quirk, 2002; Quirk & Mueller, 2008). Interestingly, the anxiety memory, i.e. the CTX–US association, is not erased during extinction. It is thought that the extinction memory and the anxiety memory would compete for expression; if the former failed to express itself, anxiety would return after extinction training (Bouton, 2002).

As reported in the general introduction, reinstatement is one of those relapse mechanisms, which can occur after another unexpected appearance of the US and elicits anxiety relapse to the extinguished stimulus (Bouton, 2002; Rescorla & Heth, 1975). So far, many studies investigated this return of fear phenomenon in humans using cue conditioning, but only few examined reinstatement effects on the return of anxiety in contextual conditioning. Glotzbach-Schoon et al. (2015) recently found anxiety relapse after reinstatement was modulated by the state anxiety of the participants. In detail, individuals with a high level of state anxiety showed differential reinstatement indicated by fear-potentiated startle responses in CTX+ compared to CTX-, whereas individuals with a low level of state anxiety demonstrated generalized reinstatement in terms of generally enhanced startle responses in both CTX+ and CTX- (Glotzbach-Schoon et al., 2015).

An effective treatment for anxiety disorder patients is exposure therapy, which is supposed to induce extinction (Craske et al., 2014; Mühlberger, Herrmann, Wiedemann, Ellgring & Pauli, 2001). However, some anxiety patients fail to respond to the treatment (Craske et al., 2014; Minnen, Wessel, Dijkstra & Roelofs, 2002). Furthermore, anxiety relapses after exposure therapy. Presumably, patients have enhanced anxiety memory or they show deficient extinction learning (Bouton, 2002). For this reason, studies on how

extinction learning can be strengthened and how the return of anxiety can be prevented may help create more effective therapies.

One promising method might be vagus nerve stimulation (Beekwilder & Beems, 2010) because it activates the brain areas involved in the formation of an extinction memory and the consolidation of this memory. In short, afferent fibers of the vagus nerve can be excited when peripheral adrenalin binds to vagal β-adrenergic receptors during an emotional experience (Miyashita & Williams, 2006). This primarily viscerosensory information is carried to the NTS and activate further brain areas (Nemeroff et al., 2006). The release of NE in key structures for the emotional memory formation including the amygdala, hippocampus, and vmPFC (Hassert et al., 2004; Roosevelt et al., 2006) is mediated either via the NTS directly or via the LC indirectly (Mello-Carpes & Izquierdo, 2013; Van Bockstaele et al., 1999). Here, particularly during fear extinction, NE released in the forebrain areas is crucial for memory formation and consolidation (Çalışkan & Albrecht, 2013; Quirk & Mueller, 2008). In this regard, VNS could be a method which specifically enhances the communication between peripheral and central nervous system during extinction learning (Çalışkan & Albrecht, 2013).

In fact, animal studies support this assumption: Peña et al. (2013) performed a conditioning experiment in rats and paired a 30s tone with an electric foot shock. As a consequence, the tone alone elicited sustained fear. Importantly, faster extinction learning and less return of fear i.e. conditioned freezing, was shown in those rats which received VNS by an implanted stimulator compared to a sham stimulated group of rats (Peña et al., 2013). Interestingly, the same effects were found for the extinction of context conditioned anxiety in a follow-up study (Peña et al., 2014) reflected by decreased freezing in the experimental cage during extinction in the VNS compared to sham stimulated rats.

In humans, patients suffering from medically intractable epilepsy were treated by an implanted vagus nerve stimulator along the cervical portion of the nerve (Couch et al., 2016). Moreover, implanted VNS serves as add-on therapy with increasing frequency for example in dementia, depression and other psychiatric disorders (Cimpianu et al., 2017). The recently developed non-invasive technique of transcutaneous vagus nerve stimulation uses the cymba conchae of the human ear as a stimulation site, which is exclusively innervated by the ABVN (Peuker & Filler, 2002). Since the ratio of afferent myelinated A beta axons is similar in ABVN and cervical vagus nerve, one can conclude that VNS and tVNS of either site elicit similar effects (Safi et al., 2016). Though somatosensory rather than viscerosensory information is linked by the ABVN to the brainstem, its central projections extend to the NTS (Nomura, 1984). Recent evidence suggests that tVNS of the ABVN elicits large alterations in the activation of the NTS, but also of other primary and higher-order targets of the vagal somatosensory information in forebrain and brainstem (Frangos et al., 2015).

Regarding fear extinction research, Burger et al. (2016) compared the effects of tVNS with sham stimulation in healthy humans by applying a classical cue conditioning paradigm. Importantly, accelerated fear extinction was found in contingency ratings (Burger et al., 2016). However, the lack of differential startle responses during acquisition did not allow any conclusions on the physiological effects of tVNS. So far, a study in humans, which investigates the effects of tVNS on extinction of contextual anxiety and relapse regarding reinstatement is still missing. Therefore, I applied virtual reality and modified the anxiety conditioning paradigm by Glotzbach-Schoon et al. (2015) according to the requirements for an appropriate tVNS and the additional investigation of reinstatement. Moreover, I used the Powerwall in the present paradigm for the

presentation of VR rather than the head-mounted display in Glotzbach-Schoon et al.'s study (2015).

Two main goals were investigated by the current study: first, the reliability of the modified virtual reality paradigm had to be tested regarding anxiety acquisition and extinction on subjective as well as on physiological level and overnight memory consolidation between acquisition, extinction and reinstatement test; second, the experimental approaches of vagus nerve stimulation on extinction in animals (Peña et al., 2014; Peña et al., 2013) should be translated to humans by examining the effects of tVNS on extinction and reinstatement providing careful control of tVNS by adding both sham stimulated participants and non-stimulated controls.

3.2 Material and methods

Within the scope of this study, Vanessa Höfling wrote her Bachelor Thesis under my supervision.

3.2.1 Participants

In total, 93 participants were recruited from the local internet platform www.wuewowas.de. Exclusion criteria were the same as in Study 1. Additionally, participants had to be naïve with respect to tVNS and the virtual environment I used was unknown for the participants. The analysis comprised data of 75 participants (41 females; age: M = 24.61 years, SD = 3.23). Eighteen additionally invited participants had to be excluded due to technical problems (N = 9), non-responder (N = 6, see Data Reduction for criteria), or quitting the experiment (N = 3). Before the experiment started, participants were randomly assigned to either verum stimulation (tVNS), sham stimulation (sham), or control (control) group. As shown in Table 3, groups did not significantly differ, neither in

gender nor in age or trait anxiety. Awareness of participants was assessed after acquisition by asking them in which context they received electric stimuli. Participants were labeled as aware when they reported the correct context–US contingency. The number of aware participants per group did not differ (Table 3). Importantly, awareness did not impact the results, which is why I also included the unaware participants into analyses. Written informed consent was given by all participants. The completion of the full experiment was reimbursed with 36 €. The study was approved by the Ethics Committee of the Medical Faculty of the University of Würzburg.

Table 3: Sample characteristics of Study 2 separated into groups.

	tVNS	**sham**	**control**	**statistics**
N (females)	25 (14)	25 (14)	25 (13)	$\chi^2(2) = 0.11$; $p = .948$
Age (*SD*)	24.9 (3.6)	24.3 (2.7)	24.6 (3.6)	$F(2,74) = 0.19$; $p = .831$
Aware	19	21	17	$\chi^2(2) = 1.75$; $p = .416$
Stimulation intensity [mA] (*SD*)	1.2 (1.1)	1.0 (0.6)	-	$F(1,49) = 0.94$; $p = .338$
Stimulation rating (*SD*)	6.9 (0.7)	6.8 (1.6)	-	$F(1,49) = 0.11$; $p = .739$
US intensity [mA] (*SD*)	2.0 (1.6)	2.7 (1.7)	2.3 (1.6)	$F(2,74) = 0.95$; $p = .393$
US rating	5.8 (0.9)	5.5 (1.0)	5.8 (1.0)	$F(2,74) = 0.81$; $p = .449$

3.2.2 Stimulus Material

Virtual reality. The equipment of the virtual environment and technological background was already used and published in several other studies (Andreatta et al., 2015b; Genheimer, 2014; Glotzbach-Schoon et al., 2013a; Tröger et al., 2012). In short, the VR environment was created with Source Engine (Valve Corporation, Bellevue, USA) and contained two distinguishable offices with similar furniture. A virtual corridor

connected both offices and served as inter-trial intervals (ITI). Participants sat 1.5 m in front of the Powerwall, which has been described in Study 1. Experimental control was established using VR-software CyberSession (CS-Research 5.6, VTplus GmbH, Würzburg, Germany; see www.cybersession.info for detailed information).

Unconditioned stimulus (US). The unconditioned stimulus was applied with the same device and method as in Study 1. In line with Study 1, I determined participants' individual pain threshold according to Andreatta et al. (2010) and increased the current intensity by 30% (Glotzbach et al., 2012). The determined mean stimulation intensity for the US was 2.34 mA (*SD* = 1.65) and the rating was 5.72 mA (*SD* = 0.97). Both did not differ between groups (all *p*s > .392; see Table 2).

Vagus nerve stimulation. The electrical stimulation of the vagus nerve on Day 2 was applied with NEMOS, a transcutaneous vagus nerve stimulator, developed by cerbomed GmbH (Erlangen, Germany). The verum tVNS was applied at the cymba conchae, the sham stimulation was administered at the helix of the outer ear (see Figure 5). The control group had the tVNS device attached to the chymba concha, however, unknown to the participants, the stimulator was never turned on. Similar to the pain threshold, participants' individual stimulation threshold was determined for all participants assigned to the verum or sham group. Thereby, the aim was to find the optimal stimulation intensity defined as eliciting a tingling sensation without pain (Busch et al., 2013). The stimulation consisted of rectangular pulses of 250 µS at 25 Hz. The procedure consisted of 10 s intervals, in which the respective stimulation was administered. Participants rated their feeling of each stimulation on an 11-point Likert scale from 0 (*no sensation*) to 3 (*slight tingling*) to 6 (*strong tingling*) to 10 (*painful*). In such a way, stimulation intensities were increased in steps of 0.1 mA until participants rated their feeling of the stimulation above 7 on the rating scale. Afterward, a decreasing series of

stimulation intensities in steps of 0.1 mA followed until participants rated the stimulation below 7. In this manner, another increasing and decreasing series of stimulation intensities were administered. The mean intensities of the two increasing and decreasing series that were rated with 7 were calculated. If the determined intensity was rated with 6 or 7 in a subsequent stimulation of 10 s, the intensity was used for the whole experiment. In the case when the ratings were too high or too low, the intensities were adapted until the participants rated the stimulation with a 6 or 7. Stimulation intensity and stimulation rating did not differ between stimulated groups, i.e. tVNS and sham (all *p*s > .337; Table 3).

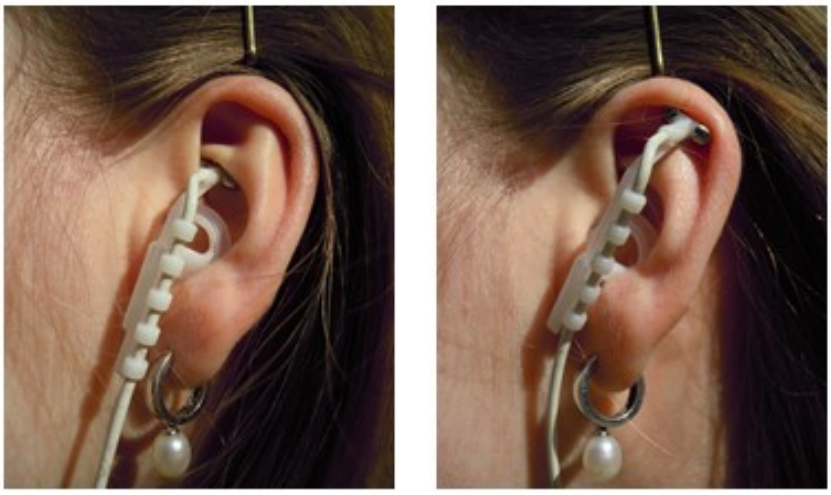

Figure 5: Stimulation sites of tVNS and sham stimulation.

The stimulator was applied to the cymba concha in the tVNS group (left) and to the helix in the sham and control group (right; Genheimer et al., 2017).

3.2.3 Measures

Questionnaires. Before the experiment, participants completed the following questionnaires: a demographic questionnaire containing age, gender, education, profession and handedness, the German versions of the ASI (Alpers & Pauli, 2001), the State-Trait Anxiety Inventory (Laux et al., 1981), the PANAS (Krohne et al., 1996). Please

note that all state questionnaires were completed at the beginning of each day of the experiment. Additionally, a questionnaire on participants' experience and opinion with the ear stimulation was assessed at the end of Day 2. Here, participants indicated their opinion on the operability of the stimulation (0 = *stimulation did not work*; 10 = *stimulation worked very well*), on the valence of the stimulation (0 = *unpleasant*; 10 = *pleasant*) as well as on their subjective conviction of the stimulation (0 = *not convinced*; 10 = *very convinced*).

Ratings. Similar to Study 1, ratings of valence, arousal, anxiety, and contingency were assessed for each context using a 100-point Likert scale presented on the Powerwall. A picture of the referring context and the rating scale were shown when the participant reported their rating verbally. The valence scale ranged from 0 (= *very unpleasant*), 50 (= *neutral*) to 100 (= *very pleasant*), the arousal and anxiety scales from 0 (= *not arousing/not anxious*) to 100 (= *very arousing/very anxious*) and the contingency scale from 0 (= *surely no US*) to 100 (= *surely US*).

Startle. White noise, which was presented binaurally with 103 dB for 50 ms via headphones, was used to elicit startle responses. At the same time, electromyographic activity (EMG) was recorded from the *M. orbicularis oculi*. Therefore, electrodes were attached below the participants' left eye. According to Blumenthal et al.'s guidelines (2005), one electrode was placed centrally below the pupil and the second electrode about 1 cm aside. The reference electrode was attached to the central forehead and the ground electrode on the left mastoid. To keep impedances of all electrodes below 10 kΩ, I prepared the respective sites on the skin with cleaning alcohol and abrasive peeling paste.

HR. As I expected relative heart rate deceleration due to verum tVNS compared to sham and control group, I recorded electrocardiogram (ECG) during extinction by two single-

use adhesive Ag/AgCl foam electrodes of 55 mm diameter by Swaromed. One electrode was placed on the right clavicle and the other on the left lower costal arch. Before the electrodes were attached, participants' skin was cleaned with alcohol. To control for the effectiveness of tVNS, I expected heart rate deceleration in the VNS group compared to the control groups during stimulation.

3.2.4 Procedure and Design

The experiment was conducted on three consecutive days separated by 24 h (Figure 6). On Day 1, all participants signed the informed consent and filled in the questionnaires. After all electrodes for physiological measures and the US electrode were attached, participants' individual pain threshold was determined according to the procedure described above. During the habituation phase, participants were teleported in the corridor in the virtual environment displayed on the Powerwall and instructed to inspect the offices by walking with a joystick. They had 2 min for inspecting each office. Afterward, I assessed baseline ratings of valence, arousal, and anxiety for each context as described above. To minimize initial startle reactivity, seven startle probes were presented with 9 to 17 s break intervals. The subsequent two acquisition phases (A1 and A2) consisted of five trials for each office resulting in a total of 20 trials. In one trial, participants were passively guided through the virtual environment, whereby two alternating pre-recorded paths of about 55 s walking either clockwise or counter-clockwise were used. One trial always started in the corridor (ca. 14 s), then participants walked through one office for 30 s and back to the corridor (ca. 8 s). Startle probes were presented randomly when participants were in the corridor (0 or 1 startle probe per trial, either before entering the office or after leaving the office) and in the offices (1 or 2 startle probes per trial, at least 6 s after entering the office). Altogether, 12 startle probes were delivered in the corridor

and 12 in each office. Between startle probes and electric stimulus, a minimum interval of 9 s was kept. For anxiety acquisition, participants could receive 0 to 2 unpredictable mildly painful US per trial, but only in the anxiety context (CTX+) and never in the safety context (CTX-). In total, 6 US were applied in each acquisition phase. To increase participants' attention on the context–US associations, the instructions for participants included a hint that it is possible to predict the electric stimuli (Schiller et al., 2010). Ratings of valence, arousal, anxiety, and contingency were assessed for CTX+ and CTX- after each acquisition phase. I counterbalanced the order of room entrances as well as the offices serving as anxiety or safety context.

On Day 2, participants completed the PANAS and STAI state questionnaires. Then the vagus nerve stimulator was attached accordingly in different groups. The stimulation threshold was determined only for the tVNS and sham group participants according to the previously described procedure (see Stimulus Material). With the individually calculated intensities, participants were stimulated for 20 min according to the stimulation rhythm of tVNS which is normally used for epilepsy patients, namely 30 s on/off cycles (cerbomed GmbH, Erlangen, Germany). Meanwhile, the EMG and HR electrodes, as well as the US electrode were attached like on Day 1. Afterward, two extinction phases (E1 and E2) were conducted, which were similar to the acquisition phases on Day 1, with the exception that no US was delivered. The numbers of startle probes stayed the same for all conditions. Importantly, vagus nerve or sham stimulation was synchronized with the 30 s, in which participants were indeed passively walking around in one office to specifically stimulate participants throughout the context duration. Ratings of valence, arousal, anxiety, and contingency were assessed before extinction started (preE) and after E1 and E2.

Day 3 again started with the completion of PANAS and STAI state. All electrodes were applied as in the previous days. Then, three unannounced USs were administered for

reinstatement of conditioned anxiety (Lonsdorf, Haaker, Fadai & Kalisch, 2014a). The procedure for the subsequent two test phases (T1 and T2) was the same as in the previous days and no further US was delivered. Again, ratings of valence, arousal, anxiety, and contingency were assessed directly after the reinstatement US (preT) and after T1 and T2.

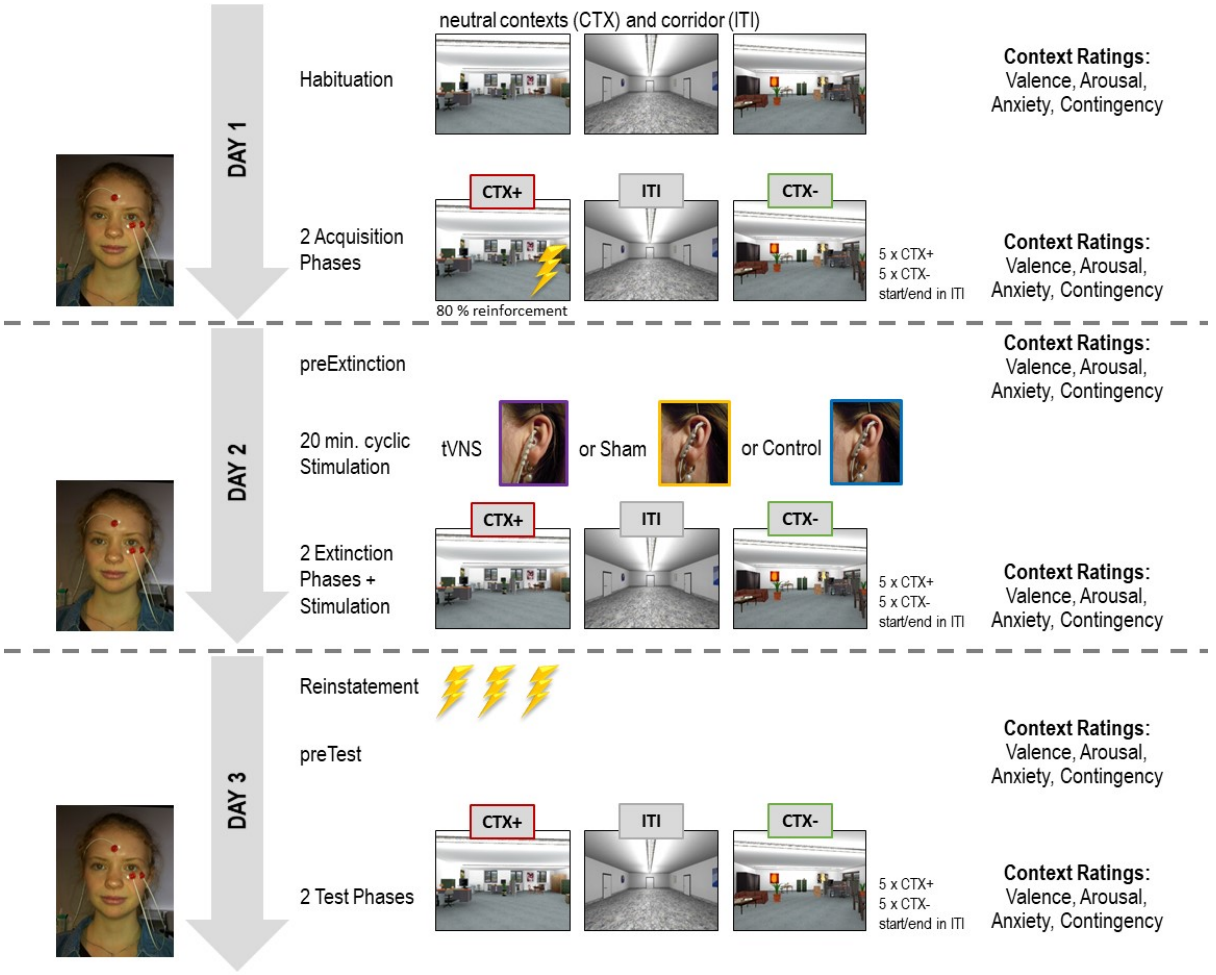

Figure 6: Experimental design of Study 2.

Shown are pictures of the three virtual contexts (anxiety context, CTX+, red; safety context, CTX-, green; and corridor, inter-trial interval, ITI, gray). Startle electrodes were attached according to the depicted photo. The experiment started on Day 1 with the habituation phase (Hab). Anxiety conditioning was performed during Acquisition 1 and 2 (A1 and A2). Day 2 started with a 20 min stimulation for the VNS (violet) and Sham (yellow) group. During both extinction phases (E1 and E2), ear stimulation occurred simultaneously to the stay in one office. No US was presented during extinction. Three US for reinstatement were administered at the beginning of Day 3. The subsequent test phases (T1 and T2) looked like the extinction without ear stimulation. Ratings of valence, arousal, anxiety, and contingency were assessed on each day between phases. Screenshots of the virtual environment were made in house.

3.2.5 Data Recordings and Data Reduction

Startle. Physiological data were continuously recorded with the Vision Recorder software (version 1.21, Brain Products Inc., Munich, Germany). The EMG sampling rate was set at 1000 Hz, an online Notch filter was applied at 50 Hz. The offline EMG data were processed with Vision Analyzer 2.1 software (Brain Products Inc., Munich, Germany). First, a low cut-off filter of 28 Hz and a high cut-off filter of 500 Hz were applied. Then, I rectified the signal, smoothed it with a moving average window of 50 ms and baseline corrected it from -50 ms to the startle probe onset (Grillon et al., 2006). Peak detection for the startle peaks was performed in a time window between 20 and 200 ms after the startle probe onset. Baseline shifts higher than 5 μV were manually scored and defined as artifacts. Those participants, whose mean startle magnitude was below 5 μV were defined as non-responders and excluded from analyses. General differences in startle responses were considered by transforming the raw data into *z*-scores within subjects and subsequently into T-scores. I interpolated missing startle responses by the mean T scores for each startle response across all participants. Subsequently, the T-scores of startles in two consecutive trials were averaged, which resulted in phases of startle responses per day, i.e. A1-A5 (acquisition), E1-E5 (extinction), and T1-T5 (test).

HR. During extinction, participants' HR was recorded with the Vision Recorder software (version 1.21, Brain Products Inc., Munich, Germany). Offline data were processed with Vision Analyzer 2.1 software (Brain Products Inc., Munich, Germany). A 30 Hz high cut-off filter was applied. R-peaks were automatically detected and manually controlled. The signal was transformed into HR in E1 and E2 for each participant and exported into SPSS for statistical analyses.

3.2.6 Statistical Analyses

Repeated measures ANOVAs were calculated for the state questionnaires PANAS as well as for STAI state and IPQ and contained the within-subject factor phase (acquisition, extinction, test) and the between-subjects factor group (tVNS, sham, control). Separate one-way ANOVAs compared the trait questionnaires ASI and STAI trait as well as stimulation and US intensities between groups.

For acquisition, extinction, and test, I calculated separate repeated-measures ANOVAs. Startle responses were analyzed by three 3 x 5 ANOVAs for each part separately with the within-subject factors context (CTX+, CTX-, ITI) and phase (A1-A5, E1-E5, and T1-T5, respectively). In parallel, the analyses of the ratings contained separate 2 x 3 ANOVAs with the within-subject factors context (CTX+, CTX-) and phase (before, after the first and after the second acquisition, extinction and test phase, respectively). All analyses on Day 2 and Day 3 included the additional between-subjects factor group (tVNS, sham, control) to investigate the effects of stimulation. For the manipulation check of the ear stimulation, HR was analyzed by a repeated-measures ANOVA containing the within-subject factor time (E1, E2) and the between-subject factor group (tVNS, sham, control).

Significant main effects and interactions were resolved by post hoc *t*-tests. When the assumption of sphericity was violated, Greenhouse-Geisser corrections were applied and Greenhouse-Geisser Epsilon (*GG-*ε) was reported. The α-level was set at 0.05 for all statistical tests.

3.3 Results

3.3.1 Questionnaires

Positive affect and antidepressant effects due to either implanted or transcutaneous vagus nerve stimulation have already been reported in several clinical studies

(Beekwilder & Beems, 2010; Fang et al., 2017; Nemeroff et al., 2006). To assess participants' affect and anxiety, I used the ASI (Alpers & Pauli, 2001), the PANAS (Krohne et al., 1996) and STAI state and trait (Laux et al., 1981).

The calculated ANOVA for ASI revealed a main effect of group ($F(2,85) = 3.96$, $p = .023$, $\eta_p^2 = .099$, for an overview see Table 4), which indicated lower anxiety sensitivity indices for participants assigned to the sham compared to both tVNS ($t(48) = 2.75$, $p = .009$) and to controls ($t(48) = 2.08$, $p = .043$). Between-group effects were neither found for PANAS nor for STAI state. The STAI state questionnaire revealed no effects of phase (all ps > .088) Interestingly, a main effect of phase was revealed for PANAS positive affect ($F(2,144) = 19.18$, $p < .001$, $\eta_p^2 = .210$) and for negative affect($F(2,144) = 4.01$, $GG\text{-}\varepsilon = .757$, $p = .031$, $\eta_p^2 = .053$). Follow-up t-tests showed higher positive affect on Day 1 compared to Day 2 ($t(74) = 5.51$, $p < .001$) and compared to Day 3 ($t(74) = 5.26$, $p < .001$). The ratings of negative affect were also higher on Day 1 compared to Day 3 ($t(74) = 2.76$, $p = .007$). The STAI trait questionnaire indicated no difference between groups ($F(2,72) = 1.91$, $p = .156$, $\eta_p^2 = .050$). The ANOVA for IPQ indicated a main effect of phase ($F(2,144) = 15.06$, $GG\text{-}\varepsilon = .824$, $p < .001$, $\eta_p^2 = .073$), but neither group nor interaction reached significance (all ps > .279). Post-hoc comparisons between phases by t-tests revealed significantly higher presence feeling on Day 1 compared to Day 2 ($t(74) = 3.81$, $p < .001$) and compared to Day 3 ($t(74) = 4.55$, $p < .001$). Rating scores between Day 2 and Day 3 did not differ ($t(74) = 1.82$, $p = .072$).

In sum, the state questionnaires PANAS and STAI did not change throughout the experimental procedure between groups, which might be due to the relatively short stimulation period (Fang et al., 2017).

Table 4: Mean scores of the questionnaires in Study 2.

Included are Anxiety Sensitivity Index (ASI), positive affect (PA), negative affect (NA) schedule (PANAS) and STAI state and trait anxiety inventory, and the comparison between groups (tVNS, sham, control).

	tVNS	**sham**	**control**	**statistics**
ASI (*SD*)	18.44 (9.14)	12.28 (6.47)	16.56 (7.98)	$F(2,74) = 3.96$; $p = .023$*
STAI trait (*SD*)	39.48 (9.37)	34.88 (8.77)	39.08 (9.54)	$F(2,74) = 1.91$; $p = .156$
PA Day 1 (*SD*)	30.48 (5.71)	32.40 (5.56)	32.08 (5.69)	$F(2,74) = 0.82$; $p = .441$
PA Day 2 (*SD*)	26.52 (6.56)	30.40 (5.58)	29.04 (8.13)	$F(2,74) = 2.07$; $p = .133$
PA Day 3 (*SD*)	25.52 (7.36)	29.80 (7.07)	28.96 (6.89)	$F(2,74) = 2.54$; $p = .086$
NA Day 1 (*SD*)	12.76 (3.31)	11.76 (2.35)	14.00 (4.94)	$F(2,74) = 2.31$; $p = .107$
NA Day 2 (*SD*)	12.24 (3.44)	11.48 (3.08)	12.72 (4.32)	$F(2,74) = 0.73$; $p = .484$
NA Day 3 (*SD*)	11.80 (2.60)	11.32 (2.06)	11.88 (2.64)	$F(2,74) = 0.38$; $p = .683$
STAI state Day 1 (*SD*)	34.96 (7.28)	33.36 (5.88)	36.56 (8.08)	$F(2,74) = 1.26$; $p = .291$
STAI state Day 2 (*SD*)	36.28 (8.04)	34.28 (7.51)	37.44 (11.56)	$F(2,74) = 0.75$; $p = .475$
STAI state Day 3 (*SD*)	35.52 (7.70)	32.68 (6.41)	35.20 (7.69)	$F(2,74) = 1.14$; $p = .326$
IPQ (*SD*) Day 1	2.90 (0.19)	2.97 (0.19)	3.44 (0.19)	$F(2,74) = 2.36$; $p = .102$
IPQ (*SD*) Day 2	2.77 (0.22)	2.62 (0.22)	2.96 (0.22)	$F(2,74) = 0.62$; $p = .543$
IPQ (*SD*) Day 3	2.49 (0.22)	2.59 (0.22)	2.92 (0.22)	$F(2,74) = 1.01$; $p = .371$

3.3.2 Stimulation conditions

Participants were assigned randomly to one of three experimental groups: Verum stimulation (VNS), sham stimulation (sham) and no stimulation (control). In order to check the comparability of all three groups, I assessed participants' subjective feeling about the stimulation efficacy, their experienced stimulation operability and the subjective pleasantness of the stimulation (valence) after the experiment. Importantly, all

groups were similarly convinced about the stimulation efficacy ($F(2,72) = 2.10$, $p = .130$, $\eta_p^2 = .055$; Table 5), however, differed in the evaluation of the experienced operability ($F(2,72) = 3.51$, $p = .035$, $\eta_p^2 = .089$) and valence of the stimulation ($F(2,72) = 4.13$, $p = .020$, $\eta_p^2 = .103$). In comparison to the control group without any stimulation, the VNS group reported a similar operability ($t(48) = 0.76$, $p = .451$) and more negative valence ($t(48) = 2.40$, $p = .020$). The sham group compared to the VNS group reported less negative valence of the stimulation ($t(48) = 2.80$, $p = .007$).

Table 5: Rating of ear stimulation in Study 2 separately for each group.

Participants were divided into three groups: Verum vagus nerve stimulation (VNS) at the cymba concha of the left ear, sham stimulation (sham) at the helix and a control group (control), in which the stimulator was applied to the cymba concha but never switched on. All participants rated their conviction of the stimulation efficacy (0 = not convinced, 10 = very convinced), the operability of the stimulation (0 = stimulation did not work, 10 = stimulation worked well), and the valence of the stimulation (0 = unpleasant, 10 = pleasant).

	tVNS	**sham**	**control**	**statistics**
Conviction (*SD*)	5.68 (1.77)	6.44 (2.12)	5.24 (2.35)	$F(2,74) = 2.10$; $p = .130$
Operability (*SD*)	7.20 (2.20)	8.36 (2.02)	6.68 (2.64)	$F(2,74) = 3.51$; $p = .035$
Valence (*SD*)	5.20 (1.98)	6.84 (2.15)	6.84 (2.78)	$F(2,74) = 4.13$; $p = .020$

3.3.3 Manipulation check of tVNS by HR

To test whether tVNS changed the HR of the participants, I calculated a repeated-measures ANOVA with the within-subjects factor time (E1, E2) and the between-subjects factor group (VNS, sham, control). Neither the main effect of time ($F(1,72) = 0.06$, $p = .804$, $\eta_p^2 = .001$), nor group ($F(2,72) = 0.44$, $p = .646$, $\eta_p^2 = .012$) nor the interaction of Time x Group ($F(2,72) = 0.00$, $p = .996$, $\eta_p^2 = .000$) reached significance. In conclusion, the current data did not indicate any changes in HR induced by tVNS. Therefore, I could not prove physiological changes due to the short and transcutaneous stimulation of the vagus nerve

I have used here. Consequently, in future studies, further methods have to be applied to test successful tVNS manipulation.

3.3.4 Acquisition of conditioned anxiety (Day 1)

Remarkably, the factor group was included in all statistical analyses of startle response, arousal, anxiety, and contingency. Since no main effect or interaction was revealed during acquisition, the statistics are not reported.

Startle. Successful anxiety acquisition in startle response was indicated by a significant main effect of context ($F(4,288) = 46.36$, $GG\text{-}\varepsilon = .818$, $p < .001$, $\eta_p^2 = .392$) and a significant interaction of Phase x Context ($F(8,576) = 4.83$, $GG\text{-}\varepsilon = .839$, $p < .001$, $\eta_p^2 = .063$). In particular, only in the last trial of the second acquisition phase (i.e., A5; see Figure 7), startle responses were potentiated in CTX+ compared to CTX- ($t(74) = 2.62$, $p = .011$). Additionally, in all acquisition phases participants showed potentiated startle responses in CTX+ as well as in CTX- compared to ITI (all *p*s < .008), which may be due to the similarity of both offices and therefore lower valence compared to the corridor.

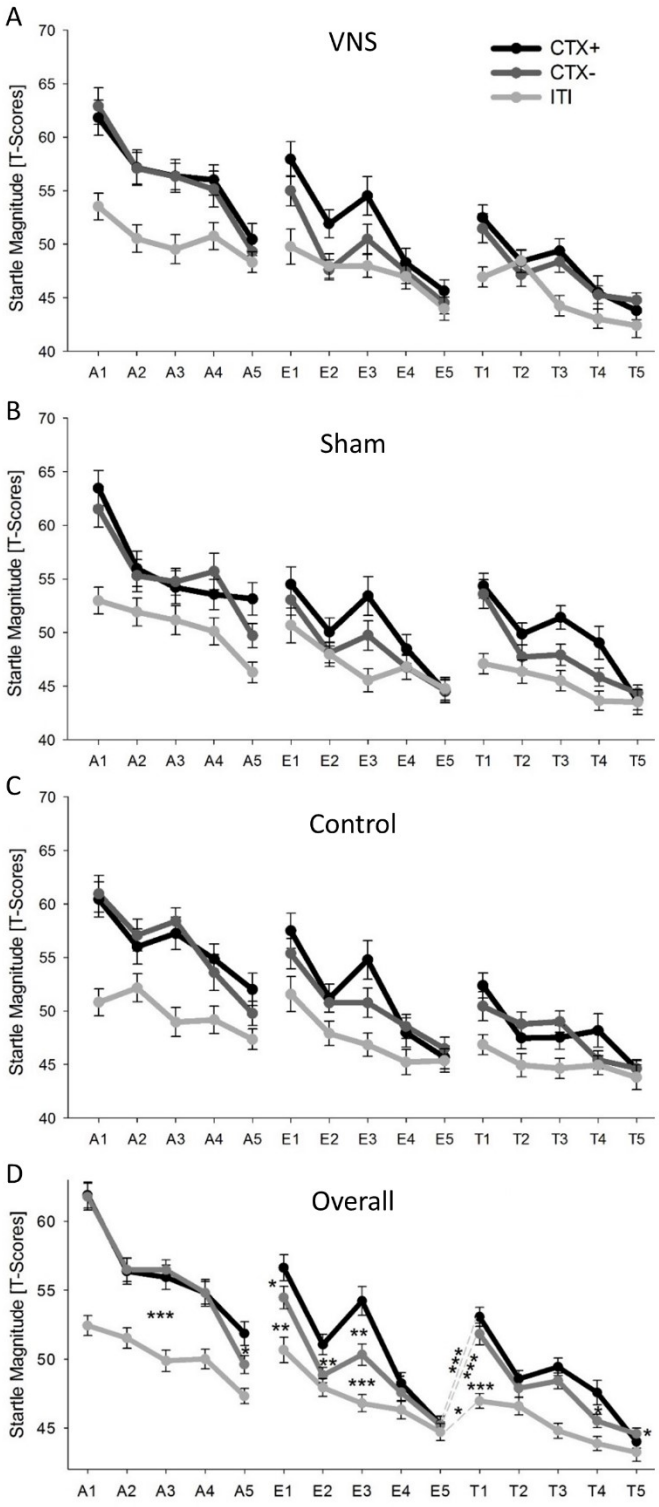

Figure 7: Startle magnitudes of Study 2.

T-scores of the startle magnitude and standard errors are depicted in the anxiety context (CTX+), safety context (CTX-) and corridor (ITI) separated by acquisition on the first day (A1-A5), extinction on the second day (E1-E5), and reinstatement/test phase on the third day (T1-T5). Each point on the x-axis depicts the mean of 2 trials. (A), (B) and (C) show startle responses of the VNS group (N = 25), Sham group (N = 25) and control group (N = 25), respectively. (D) depicts the startle responses of all (N = 75) participants (Genheimer, Andreatta, Asan & Pauli, 2017). *: $p < .05$; **: $p < .01$; ***: $p < .001$.

Valence. After habituation, participants rated the valence of both context on a similar level ($F(1,72) = 0.47$, $p = .496$, $\eta_p^2 = .006$). During acquisition, a significant main effect of context ($F(1,72) = 8.11$, $p = .006$, $\eta_p^2 = .101$) and a non-significant interaction Phase x Context: ($F(1,72) = 0.01$, $p = .940$, $\eta_p^2 = .000$) revealed overall more unpleasantness in CTX+ compared to CTX- and a very fast change in the valence ratings of the contexts (see Figure 8A). Though a significant interaction of Phase x Group ($F(2,72) = 3.21$, $p = .046$, $\eta_p^2 = .082$) was observed, post-hoc *t*-tests did not reach significance (all *p*s ≤ .063).

Arousal. Participants rated the level of arousal similar in both contexts after habituation ($F(1,72) = 0.00$, $p = .963$, $\eta_p^2 = .000$). During acquisition, the arousal ratings to each context changed rapidly indicated by a significant main effect of context ($F(1,72) = 19.78$, $p < .001$, $\eta_p^2 = .215$) and a non-significant interaction Phase x Context ($F(1,72) = 0.82$, $p = .368$, $\eta_p^2 = .011$). As expected, arousal was higher in CTX+ compared to CTX- (see Figure 8B).

Anxiety. After habituation, the anxiety ratings were similar for both contexts ($F(1,72) = 0.09$, $p = .771$, $\eta_p^2 = .001$). Again, successful and fast differential anxiety conditioning was indicated by a significant main effect of context ($F(1,72) = 19.97$, $p < .001$, $\eta_p^2 = .217$) and a non-significant interaction of Phase x Context ($F(1,72) = 1.26$, $p = .265$, $\eta_p^2 = .017$) revealing higher anxiety in CTX+ compared to CTX- (see Figure 8C).

Contingency. During acquisition, a significant main effect of context ($F(1,72) = 68.93$, $p < .001$, $\eta_p^2 = .489$) as well as a significant interaction of Phase x Context ($F(1,72) = 22.19$, $p < .001$, $\eta_p^2 = .236$) demonstrated higher contingency ratings for CTX+ compared to CTX- for both A1 ($t(74) = 5.83$, $p < .001$) and A2 ($t(74) = 8.65$, $p < .001$; see Figure 8D). Hence, successful anxiety conditioning can be concluded.

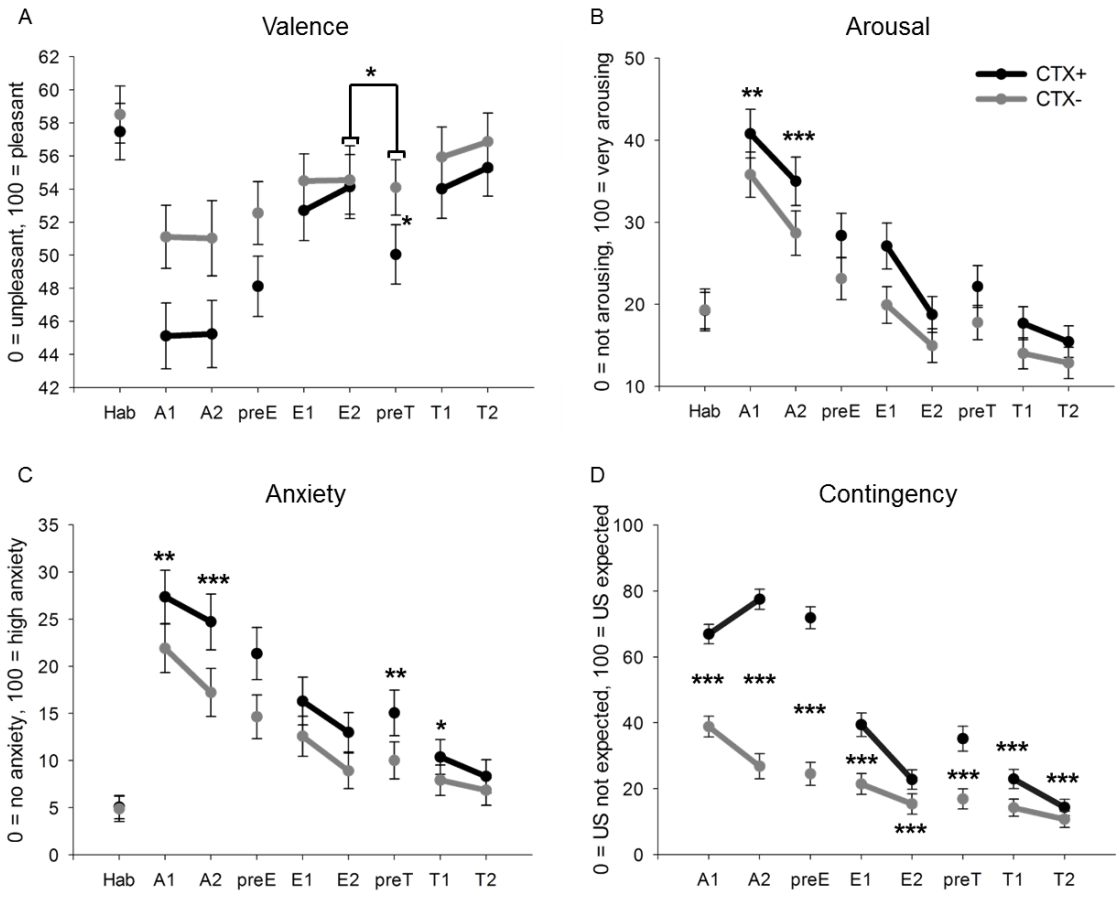

Figure 8: Ratings for context conditioning in Study 2.

Data for each rating were pooled across all three groups and are shown in one overall graph (N = 75). Circles (with standard errors) depict valence (A), arousal (B), anxiety (C), and contingency (D) ratings for anxiety context (CTX+) and safety context (CTX-). X-Axes show the time of the rating: After habituation (Hab), after Acquisition 1 (A1), and Acquisition 2 (A2) for Day 1, before (preE) and after Extinction 1 (E1) as well after Extinction 2 (E2) for Day 2, and for Day 3 before (preT) and after Test 1 (T1) and Test 2 (T2; Genheimer et al., 2017). *: $p < .05$; **: $p < .01$; ***: $p < .001$

3.3.5 Extinction of conditioned contextual anxiety (Day 2)

For extinction on Day 2, all participants were again guided through both virtual offices, however, no USs were administered anymore. All analyses of startle responses, arousal and anxiety ratings, which included the between-subjects factor group were not significant (all *ps* > .273) and therefore not reported in the following results section.

Startle. The ANOVA revealed a significant main effect of context ($F(2,144) = 28.83$, $p < .001$, $\eta_p^2 = .286$) and a significant interaction of Phase x Context ($F(8,576) = 5.43$, $GG\text{-}\varepsilon = .819$, $p < .001$, $\eta_p^2 = .070$). Follow-up post-hoc *t*-tests returned potentiated startle responses in CTX+ compared to CTX- during E1 ($t(74) = 2.51$, $p = .014$), E2 ($t(74) = 2.75$, $p = .007$), and E3 ($t(74) = 3.39$, $p = .001$), but not during E4 and E5 (all *p*s > .432; Figure 7). Therefore, I conclude successful physiological extinction of conditioned anxiety indicated by startle responses.

Valence. For the valence ratings prior to extinction (preE) the main effect of context just failed to reach significance ($F(1,72) = 0.53$, $p = .053$, $\eta_p^2 = .051$). A significant interaction of Context x Group ($F(2,72) = 3.64$, $p = .031$, $\eta_p^2 = .092$) demonstrated more negative overall valence ratings in CTX+ compared to CTX- only in the control group ($t(24) = 2.63$, $p = .015$). Interestingly, a significant main effect of group ($F(2,72) = 4.21$, $p = .019$, $\eta_p^2 = .105$) indicated more negative valence ratings in the tVNS compared to both the sham ($t(48) = 2.14$, $p = .037$) and the control group ($t(48) = 3.12$, $p = .003$). This is consistent with the more negative valence ratings of the transcutaneous vagus nerve stimulation itself. Further effects of phase and context were not significant (all *p*s > .367, Figure 9A-C).

Arousal. Before extinction started (preE), a main effect of context ($F(1,72) = 7.40$, $p = .008$, $\eta_p^2 = .093$) revealed significantly higher arousal in CTX+ compared to CTX-. During extinction, a significant interaction of Phase x Context ($F(1,72) = 5.17$, $p = .026$, $\eta_p^2 = .067$) indicated higher arousal in CTX+ after E1 ($t(74) = 4.42$, $p < .001$) but also after E2 ($t(74) = 2.91$, $p = .005$). For this reason, I conclude the existence of extinction learning, however with incomplete success for arousal ratings (Figure 9D-F).

Anxiety. At pre-extinction, a significant main effect of context ($F(1,72) = 10.34$, $p = .002$, $\eta_p^2 = .126$) indicated higher anxiety ratings in CTX+ compared to CTX-. During extinction,

a significant main effect of context ($F(1,72) = 11.73$, $p = .001$, $\eta_p^2 = .140$), but the lack of the interaction of Phase x Context ($F(1,72) = 0.08$, $p = .783$, $\eta_p^2 = .001$) demonstrated overall enhanced anxiety ratings in CTX+ compared to CTX-, which points out insufficient extinction learning (Figure 9G-I).

Contingency. A significant main effect of context ($F(1,72) = 81.76$, $p < .001$, $\eta_p^2 = .532$), but no group effects (all *p*s > .149) at pre-extinction demonstrated higher US expectancy in CTX+ compared to CTX-. This shows the maintenance of associations previously learned in all groups. During extinction, a main effect of context ($F(1,72) = 28.16$, $p < .001$, $\eta_p^2 = .281$) and an interaction of Phase x Context ($F(1,72) = 20.04$, $p < .001$, $\eta_p^2 = .218$) confirmed higher contingency ratings in CTX+ compared to CTX- after for E1 ($t(74) = 5.45$, $p < .001$), and still after E2 ($t(74) = 4.24$, $p < .001$). Therefore, extinction learning occurred but was incomplete (Figure 9J-L).

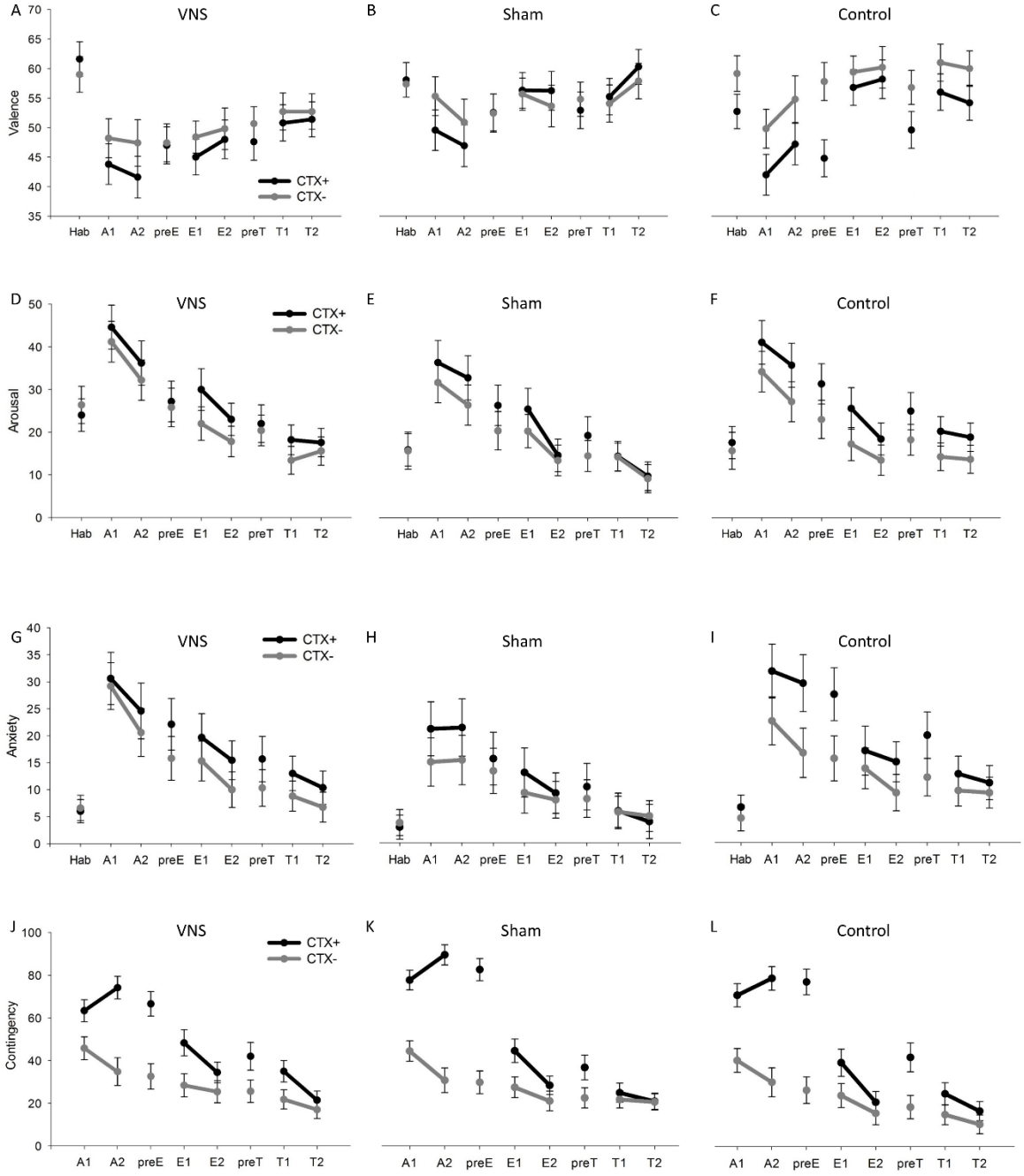

Figure 9: All ratings of Study 2 separated into groups.

Left panel: VNS group (N = 25), middle panel: Sham group (N = 25), right panel: Control group (N = 25). A-C: Valence ratings (0 = very unpleasant, 100 = very pleasant), D-F: Arousal ratings (0 = not arousing, 100 = very arousing), G-H: Anxiety ratings (0 = not anxious, 100 = very anxious), J-L: contingency ratings (0 = surely no US, 100 = surely US). Hab = Habituation, A1 = Acquisition 1, A2 = Acquisition 2, preE = pre-Extinction, E1 = Extinction 1, E2 = Extinction 2, preT = pre-Test, T1 = Test 1, T2 = Test 2 (Genheimer et al., 2017).

3.3.6 Reinstatement of conditioned anxiety (late Day 2 vs. early Day 3)

At the beginning of Day 3, three unannounced US were delivered to induce reinstatement effects. Thereby, a general increase in responses from the end of extinction to the first test trial would demonstrate general reinstatement, indicated by a significant effect of phase; in contrast, differential responses for CTX+ and CTX- at the first test trial would show differential reinstatement indicated by a significant Phase x Context interaction. Since the test for reinstatement requires successful extinction, i.e. no difference between CTX+ and CTX- at the end of extinction, I only could test reinstatement for startle responses and valence ratings. Since all effects which included the factor group were not significant (all *p*s > .058), those effects will not be reported below.

Startle. For the analyses of reinstatement in startle responses, the calculated ANOVA returned significant main effects of phase ($F(1,72) = 56.89$, $p = .001$, $\eta_p^2 = .440$) and context ($F(2,144) = 19.21$, $p = .001$, $\eta_p^2 = .211$) as well as an interaction of Phase x Context ($F(2,144) = 13.48$, $p < .001$, $\eta_p^2 = .158$; Figure 7). The interaction was followed up by post-hoc *t*-tests and revealed return of anxiety for all conditions. In particular, significantly potentiated startle responses during the first test trials as compared to the last extinction trials were found for CTX+ ($t(74) = 7.78$, $p < .001$), CTX- ($t(74) = 6.17$, $p < .001$) and ITI ($t(74) = 2.60$, $p = .011$). Importantly, no differences were found between CTX+ and CTX- ($t(74) = 1.10$, $p = .247$) during the first test trials. However, potentiated startle responses to CTX+ ($t(74) = 5.64$, $p < .001$) and CTX- ($t(74) = 5.26$, $p < .001$) compared to ITI indicated general reinstatement.

Valence. Reinstatement test revealed a significant main effect of phase ($F(1,72) = 4.24$, $p = .043$, $\eta_p^2 = .056$) and a significant interaction of Phase x Context ($F(1,72) = 4.75$, $p = .033$, $\eta_p^2 = .062$; Figure 8). The main effect of context returned no significance ($F(1,72) = 2.15$, $p = .147$, $\eta_p^2 = .029$). Follow-up post hoc *t*-tests of the interaction indicated no

difference between valence ratings of CTX+ and CTX- at the end of extinction ($t(74) = 0.27$, $p = .788$), but more negative valence in CTX+ compared to CTX- after reinstatement ($t(74) = 2.08$, $p = .041$). In sum, valence ratings demonstrated differential reinstatement, i.e. a differential return of conditioned valence after reinstatement.

3.3.7 Re-extinction (Day 3)

The re-extinction or test phase was analyzed by startle responses on Day 3 and ratings assessed after the first and the second test phase. Since neither main effect of group nor interactions with group returned any significant results in the startle response, arousal and anxiety ratings (all *p*s > .079), statistics are not reported below.

Startle. Analyses of startle responses revealed a main effect of context ($F(2,144) = 42.56$, GG-ε = .733, $p < .001$, $\eta_p^2 = .372$) and an interaction of Phase x Context ($F(8,576) = 4.54$, GG-ε = .846, $p < .001$, $\eta_p^2 = .059$; Figure 7). In fact, startle responses were potentiated in CTX+ compared to ITI in T1 ($t(74) = 6.94$, $p < .001$), T2 ($t(74) = 2.30$, $p = .024$), T3 ($t(74) = 6.33$, $p < .001$), and T4 ($t(74) = 3.89$, $p < .001$), but not in T5 ($t(74) = 1.06$, $p = .291$) and in CTX- compared to ITI in T1 ($t(74) = 5.54$, $p < .001$), T3 ($t(74) = 5.03$, $p < .001$), T4 ($t(74) = 2.48$, $p = .015$), and T5 ($t(74) = 2.04$, $p = .045$), but not in T2 ($p = .136$). Startle responses did not significantly differ between CTX+ and CTX- in T1, T2, T3 and T5 (all *p*s > .149), but in T4 ($t(74) = 2.36$, $p = .021$). In sum, re-extinction of the induced general reinstatement effect could be revealed.

Valence. Neither effect of context nor interaction of Phase x Context (all *p*s > .184; Figure 9A-C) indicated successful re-extinction of the induced differential reinstatement effect. Please note that the significant interaction of Phase x Group ($F(2,72) = 4.88$, $p = .010$, $\eta_p^2 = .119$) demonstrated increasing valence ratings from T1 to T2 in the sham group.

Arousal. Insufficient re-extinction was revealed by testing the arousal ratings. In particular, the significant main effect of context ($F(1,72) = 9.91$, $p = .002$, $\eta_p^2 = .121$), but the absent interaction of Phase x Context ($F(1,72) = 0.78$, $p = .380$, $\eta_p^2 = .011$; Figure 9D-F) revealed generally higher arousal ratings in CTX+ compared to CTX- during the test phase.

Anxiety. Neither the main effect of context ($F(1,72) = 3.90$, $p = .052$, $\eta_p^2 = .051$) nor the interaction of Phase x Context ($F(1,72) = 2.02$, $p = .159$, $\eta_p^2 = .027$; Figure 9G-I) reached significance level. As a conclusion, re-extinction was successful.

Contingency. The analyses of contingency ratings during the re-extinction returned main effects of group ($F(2,72) = 4.56$, $p = .014$, $\eta_p^2 = .112$), context ($F(1,72) = 18.91$, $p < .001$, $\eta_p^2 = .208$), and an interaction of Phase x Context ($F(1,72) = 12.11$, $p = .001$, $\eta_p^2 = .144$; Figure 9J-K). Following up the interaction, post-hoc *t*-tests revealed higher contingency ratings for CTX+ compared to CTX- after preT ($t(74) = 4.76$, $p < .001$), after T1 ($t(74) = 4.55$, $p < .001$), and after T2 ($t(74) = 2.87$, $p = .005$). Post-hoc *t*-tests of the main effect of group indicated lower contingency ratings in the sham group compared to the tVNS group ($t(48) = 3.42$, $p = .002$) and compared to controls ($t(48) = 2.13$, $p = .040$). tVNS and control group did not differ ($t(48) = 1.05$, $p = .301$). Hence, I found incomplete re-extinction and stimulation effects, which were unrelated to conditioning.

3.3.8 Exploratory Analyses

Startle and stimulation intensity. Since the stimulation intensity could have impacted reinstatement of physiological startle responses, I calculated difference scores for startle responses between T1 and E2 for CTX+, CTX- and ITI, respectively in an exploratory manner. Then, for both stimulated groups, i.e. tVNS and sham, I performed separate Pearson's correlations of stimulation intensity and differences in startle responses. In the

tVNS group, a positive correlation for CTX- ($r(23) = 0.441$, $p = .028$) demonstrated that the higher the stimulation intensity was, the larger was the difference of startle magnitude between T1 and E2 indicating greater reinstatement. Stimulation intensity and CTX+ ($r(23) = 0.339$, $p = .098$) or ITI ($r(23) = -0.170$, $p = .415$) did not significantly correlate in the tVNS group. In the sham group, none of the correlations returned significant effects (all ps > .487).

Valence and stimulation intensity. In parallel to prior analysis, I also correlated the stimulation intensity with the difference scores for valence ratings of preT and E2 for CTX+ and CTX-, respectively. However, Pearson's correlations of stimulation intensity and contexts correlated with each other in neither the tVNS nor the sham group (all ps > .176).

Reinstatement and state anxiety. Evidence suggests that state anxiety influences reinstatement (Glotzbach-Schoon et al., 2015). In particular, startle responses revealed differential reinstatement in high state anxious participants and generalized reinstatement in low anxious participants. Glotzbach-Schoon et al. (2015) used the median split of STAI state scores (split value was 34) on Day 3 just before reinstatement took place. This resulted in two groups of 11 low anxious (STAI state score: $M = 30.00$, $SD = 3.00$) and 10 high anxious (STAI state score: $M = 42.40$, $SD = 10.53$) participants. Therefore, I similarly calculated the median split of STAI state scores (split value was 33) on Day 3 right before reinstatement for the current study. One group of 37 low state anxious participants ($M = 28.68$, $SD = 3.21$) and another group with 38 high state anxious participants ($M = 40.11$, $SD = 5.52$) were revealed. For analyses, I calculated an ANOVA including the within-subjects factors context (CTX+, CTX-, ITI) and time (E5, T1) and the between-subjects factor state anxiety (low state anxiety, high state anxiety). Significant main effects of context ($F(2,146) = 19.50$, $p < .001$, $\eta_p^2 = .221$) and phase ($F(1,73) = 55.57$, $p < .001$, $\eta_p^2 = .432$) as well as a significant interaction of Context x Time ($F(2,146) = 13.94$,

$p < .001$, $\eta_p^2 = .160$) were revealed. Importantly, the 3-way interaction of Context x Time x State Anxiety turned out to be marginally significant ($F(2,146) = 2.61$, $p = .077$, $\eta_p^2 = .034$; see Figure 10) and was exploratory further dissolved by post-hoc *t*-tests separated for the low and the high anxious group. In E5, no significant differences were shown between CTX+, CTX- and ITI (all *p*s > .113). Interestingly, during T1, groups differed significantly: Similar startle magnitudes for CTX+ and CTX- ($t(36) = 0.18$, $p = .862$) in the low state anxiety group indicated generalized reinstatement, though potentiated startle responses were revealed for CTX+ compared to ITI ($t(36) = 5.23$, $p < .001$) and for CTX- compared to ITI ($t(36) = 4.63$, $p < .001$). In contrast, in the high state anxiety group marginally potentiated startle responses in CTX+ compared to CTX- ($t(37) = 1.77$, $p = .086$) suggested differential reinstatement. In line with low state anxious participants, CTX+ ($t(37) = 4.54$, $p < .001$) and CTX- ($t(37) = 3.20$, $p = .003$) elicited higher startle magnitudes compared to ITI. Regarding state anxiety effects on reinstatement, current results replicated the findings of a prior study (Glotzbach-Schoon et al., 2015). Glotzbach-Schoon et al. (2015) discussed the mood-congruent memory effect as a possible explanation, a phenomenon that emotionally experienced material will be remembered more easily in the same emotional situation (Lewis & Critchley, 2003). Here, the emotional content of CTX+ might fit the emotional content of the anxiety memory in high state anxious participants and therefore boost the return of anxiety in CTX+. In contrast, low anxiety could be more closely related to the extinction memory and therefore boost the expression of extinction memory, which resulted in lower reinstatement. As a caveat, in Glotzbach-Schoon et al. (2015) and the current study, median splits were used to separate high and low anxious participants. For more evidence of this effect, future studies that specifically manipulate state anxiety necessary.

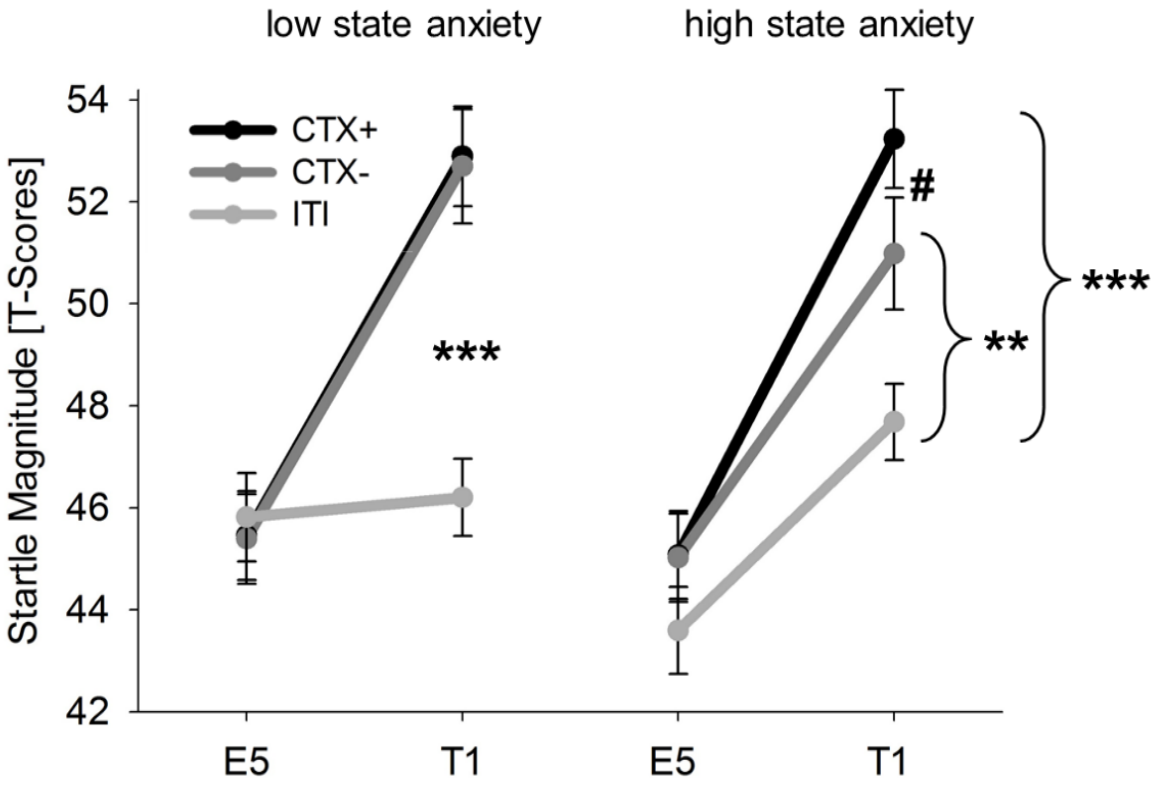

Figure 10: Reinstatement effects on startle magnitude in Study 2.

The figure illustrates reinstatement effects separated for low (left panel) and high (right panel) state anxious participants (Genheimer et al., 2017). #: $p < .09$; **: $p < .01$; ***: $p < .001$.

3.4 Discussion

The first goal of the current study was to experimentally demonstrate the return of conditioned anxiety, i. e. reinstatement, in a three days virtual reality context conditioning paradigm. Due to memory consolidation processes, the examination of acquisition, extinction, and reinstatement on different experimental days is therefore crucial (see Lonsdorf et al., 2017). Second, I aimed to translate the positive effects of implanted VNS on extinction and return of anxiety in rats (Peña et al., 2013) to humans by using tVNS. The effectiveness of tVNS during extinction would present a new treatment option for anxiety disorder and PTSD patients.

Concerning the first aim of this study, acquisition of contextual anxiety regarding stronger physiological (startle responses) and verbal (ratings of valence, arousal, anxiety, and contingency) measures in the anxiety compared to the safety context were successfully revealed. After overnight consolidation of the learned associations, at the beginning of Day 2, these effects were still present before extinction, which corroborates consolidated anxiety memory traces. Subsequent extinction was successful, indicated by startle responses and valence ratings, however, stayed incomplete for arousal, anxiety and contingency ratings. Previously, several other studies (Glotzbach-Schoon et al., 2013c; Tröger et al., 2012) have also found a similar dissociation between different anxiety measures in extinction learning. This issue was discussed in a recent review by Haaker, Golkar, Hermans, and Lonsdorf (2014a). Evidently, the acquisition of the CS-US memory association is created faster than the CS-noUS memory association built up during extinction. Regarding evolution, the accelerated creation of an anxiety memory and therefore a fight or flight response in ambiguous situations might have been a pivotal strategy for survival. In future studies, successful extinction in particular for ratings could be obtained by prolonged extinction in terms of more extinction trials.

After successful extinction in startle responses and valence ratings, diverging effects on reinstatement were found for both measures. In particular, differential reinstatement (Haaker et al., 2014a) was indicated by valence ratings in terms of diminished valence in CTX+ only. In contrast, I found generalized reinstatement for startle responses, which was emphasized by potentiated startle magnitudes in both CTX+ and CTX- compared to ITI. Supportively, Glotzbach-Schoon et al. (2015), who used a similar VR paradigm, and Haaker et al. (2013), who used pictures as different contexts, demonstrated in their respective study differential reinstatement in anxiety ratings and generalized reinstatement in startle responses. Reinstatement of conditioned anxiety is one of the

phenomena which corroborates the idea that during extinction learning, anxiety memories are not erased (Bouton, 2002). Remarkably, aversive life events, which are remembered very well, play a crucial role in evolution (Kindt, Soeter & Vervliet, 2009). In their two-system framework theory, Hamm and Weike (2005), as well as LeDoux and Pine (2016), described a cognitive level of fear, which can be assessed by ratings, and a defensive survival circuit, which induces changes on physiological responses to threat and defensive behavior.

This model can account for the divergence in startle data and valence ratings: the former indicates a response driven by a low-level, reflexive defensive circuit while the latter demonstrates a response driven by a higher level cognitive system (LeDoux & Pine, 2016). The divergence is thought to be a result of these two systems operating independently from each other (LeDoux & Pine, 2016). Though I changed several parameters in the current experiment compared to the study by Glotzbach-Schoon et al. (2015), for instance, the use of a Powerwall instead of a head-mounted display, the number of USs for reinstatement and the duration and number of trials, the findings are highly comparable. To demonstrate the validity of study designs, Richter (2017) recently emphasized the importance of systematic heterogenization of experimental protocols. In this way, the design of the current study provides substantiated evidence for a reliable and valid three days contextual anxiety conditioning paradigm aiming at the investigation modulations of return of anxiety induced by reinstatement. Additionally, the use of VR has been established as an ergonomic and elegant tool for the investigations of contextual anxiety under highly controlled and mostly naturalistic experimental conditions (Baas et al., 2004; Bohil et al., 2011; Glotzbach-Schoon et al., 2013a, 2015).

My second aim of this study was to translate the results of accelerated and stabilized extinction memory in rats by VNS (Peña et al., 2013) to humans. In short, the mechanism

of VNS is described by an increase in NE release in brain areas like mPFC and BLA, which are highly involved in extinction learning (Milad & Quirk, 2012). Peña et al. (2014) described the consequences of NE release with facilitated neural plasticity in the respective brain areas, which resulted in stronger neural IL-BLA pathways and consequently in stronger extinction memory traces (Peña et al., 2014). Notably, VNS is also suggested to enhance the extinction of drug-seeking behavior in rats (Childs, DeLeon, Nickel & Kroener, 2017).

In the current study, I did not find any reliable effect of tVNS neither on extinction nor on the reinstatement of contextual anxiety. Both physiological and verbal measures of anxiety yielded either complete or incomplete extinction to CTX+, suggesting the lack of tVNS effects in humans. Although successful extinction was found in startle responses and valence ratings, I could not observe tVNS effects on any of the examined parameters. Therefore, I argue that tVNS – as realized here – did not effectively improve extinction or prevent the return of anxiety.

The difference in stimulation methodology between animals and humans, i.e. VNS versus tVNS, is presumably the most apparent reason for divergent results and the lack of translational success. Obviously, in animals as well as in humans surgery for the direct stimulation of the neck portion of the vagus nerve is necessary. Therefore, VNS can only be investigated in patients, who wear an implanted stimulator for medical reasons (George et al., 2007), e.g. epilepsy. Hence, the implementation of tVNS in human studies without required surgery is a very promising and clinically highly relevant approach to improve fear extinction. Though one has to note that the electrical stimulation of the auricular branch of the vagus nerve might be weaker than the direct stimulation. The results of the current study are a very important step towards the optimal stimulation

parameters and experimental designs. From here, information about the so far very limited knowledge of the most effective stimulation parameters can be gained.

Frangos et al. (2015) measured fMRI while cymba conchae was constantly stimulated and found that brain areas like NTS and amygdala need at least seven minutes to be activated. I used a 20 min interval stimulation protocol consisting of 30 s on-off cycles and revealed no tVNS effects. One option is that my tVNS interval stimulation protocol was still too short to be effective. This is suggested by studies that reported physiological effects but applied tVNS a few hours per day for several weeks (Fang et al., 2017). Besides stimulation duration, the timing of the stimulation might also be an important factor for effective tVNS. The precise timing of VNS with a motor response of a sensory event induced cortical plasticity in animals (Kilgard, 2012). For this reason, I exactly paired the onset of an exact 30 s stimulation interval with participants entering into a virtual office during extinction. For the most effective stimulation, future studies should incorporate this procedure.

Since tVNS did not cause any significant changes in HR, future studies should include at least one reliable indicator for vagal activity. Heart rate variability (Yuen & Sander, 2017) or the measure of pupil dilation (Jodoin, Lespérance, Nguyen, Fournier-Gosselin & Richer, 2015) and/or analgesic effect (Busch et al., 2013) could be used as the aforementioned indicator. With a more valid manipulation check, future studies are warranted first to prove the effectiveness of tVNS and second to be able to draw substantial conclusions of the effectiveness of tVNS on extinction learning and return of anxiety.

Additionally, the respective study protocol to induce conditioned fear or anxiety might have been relevant for the interpretation of the current results. While most animal studies including Peña et al. (2013) performed a single cue or context conditioning by using either

one stimulus (for instance a tone) or a context (for instance a cage) associated with the aversive US during acquisition, most human studies are carried out via a differential conditioning procedure in which one of two cues or contexts (for example one of the offices) becomes associated with the aversive US (anxiety context) but not the other (safety context). A meta-analysis by Lissek et al. (2005) suggested that such differences in the paradigm might be crucial for the study outcome. The application of single stimulus or context conditioning protocols in humans have to be pondered to stay closer to animal experiments and to translate animal VNS research more easily to human tVNS research.

So far, only a few studies in humans investigated the effects of VNS or tVNS on extinction learning. Patients suffering from a conventional treatment-resistant psychiatric disorder like OCD, PD or PTSD but wore an implanted VNS were examined by George et al. (2008). Interestingly, in one-third of the patients, anxiety symptoms improved due to VNS.

TVNS could be a reasonable alternative for the implanted VNS (Safi et al., 2016). Safi et al. (2016) confirmed that a relatively high number of myelinated afferent A-beta fibers, which are thought to mediate the stimulation of invasive VNS, are also present in the human ABVN. In line with this are the results by Burger et al. (2016), who used a cue conditioning paradigm and demonstrated lower ratings of online contingency during extinction in the tVNS compared to a sham stimulated group. Not surprisingly, those learning curves differed mainly at the beginning of the extinction phase. In sum, the effectiveness of tVNS on cognitive extinction learning in humans is supported by the results of this study (Burger et al., 2016), but further specifications are required. In contrast, my results showed a tendency for higher contingency ratings in tVNS compared to sham stimulated participants. Again, the discrepancy of those results can be explained by methodological differences between the two studies, i.e. different stimulation

intensities, fear vs. anxiety conditioning paradigms, or online vs. offline ratings (Roosevelt et al., 2006). In detail, the current study contained only two offline contingency ratings, i.e. after each phase of extinction, which could have been too few to assess group differences between tVNS and sham during the initial extinction trials. Additionally, the stimulation intensities differed between Burger et al. (2016) and my study. While Burger et al. (2016) used the same intensity of 0.5 mA for all participants, I adjusted the stimulation intensity individually ending up with a mean intensity of 1.2 mA. Hence, the exploratory analyses of the current study showed impaired safety learning during extinction indicated by the fact that a higher stimulation intensity in the tVNS group was associated with higher startle magnitudes in the safety context after reinstatement. So, the distraction by the high stimulation intensity could have negatively effected extinction learning. One strength of the current study is the inclusion of two control groups for tVNS. While previous studies mainly compared a verum tVNS with a sham stimulated group, I added another control group without stimulation. Therefore, the discussion of unspecific effects of the stimulation is possible. Notably, before extinction started the control group rated CTX+ as more unpleasant compared to CTX-. This difference was not significant in neither of the stimulated groups. Two explanations are possible: first, the 20 min interval stimulation before extinction in both stimulated groups might have made the valence ratings of CTX+ and CTX- more similar compared to the control group that directly started with the valence ratings; second, I am convinced that context effects play a crucial role in extinction. While the control group perceived the exact same context as during acquisition, the stimulated groups felt an additional tingling of the ear stimulation, which constituted an additional part of the context and making acquisition and extinction context dissimilar. Future studies should be aware of those context effects and carefully control them (Burger et al., 2017).

At the moment, there are many discussions on how extinction learning and exposure-based therapy could be improved. Among others, pharmacological approaches (Ledgerwood et al., 2004; Ressler et al., 2004; Richardson et al., 2004), stress induction (Merz et al., 2014), as well as behavioral approaches are debated. The importance of the latter was underlined by Pittig, van den Berg, and Vervliet (2016) including procedural strategies during extinction, for instance, multiple context exposure (Shiban, Pauli & Mühlberger, 2013), and flanking strategies before and after extinction, for example, the induction of positive affect which is thought to positively influence safety learning (Meulders, Meulders & Vlaeyen, 2014). Regarding this distinction, tVNS might serve as a procedural strategy by pairing the stimulation exactly with the extinction stimulus (Peña et al., 2013), but also as a flanking strategy by activating the extinction network before extinction learning (Frangos et al., 2015). For this reason, the use of tVNS during extinction learning and potentially also during exposure-based therapy is a very promising new approach and needs further investigation.

In conclusion, exposure-based therapy for anxiety disorders can be experimentally modeled by extinction learning after conditioning (Hofmann, 2008). For these investigations, experimental paradigms like the three-day virtual reality context conditioning paradigm used in the current study are highly required. With such paradigms, follow-up research on the facilitation of extinction learning and the prevention of the return of anxiety by vagus nerve stimulation is possible and fosters the improvement of exposure-based therapy.

4 Study 3: tVNS and Pain

4.1 Introduction

According to the International Association for the Study of Pain (IASP), pain is an unpleasant emotional experience or an unpleasant sensory experience, which is '*associated with actual or potential tissue damage, or described in terms of such damage*' (retrieved from http://www.iasp-pain.org/Education/Content.aspx?ItemNumber=1698, last update 2017). Nineteen percent of European adults suffer from chronic pain, which is pain which extended over at least 6 months, e.g. pain in the back, knee or head (Breivik, Collett, Ventafridda, Cohen & Gallacher, 2006). In contrast to chronic pain, nearly everybody experiences acute pain, which is mostly the consequence of nociceptive activation. The pain perception serves as an important alarm signal to the body, triggers behavioral changes, and therefore ensures the integrity of the body (see Scholz & Woolf, 2002). Patients with congenital analgesia, which is characterized by pain insensitivity, suffer from physical, mainly orthopedic long-term consequences of non-treated injuries and die prematurely (Losa et al., 1989). In sum, on the one hand, many people severely suffer from pain, but on the other hand, despite its unpleasantness, pain is a relevant alarm signal for the maintenance of our physical integrity.

Depending on the properties of the painful stimulation, mechanical, thermal or chemical nociceptors are activated and mediate the information about pain sensation by primary afferents via the spinal cord, brain stem and thalamus to subcortical structures (National Research Council, 2009). Most nociceptors are associated with unmyelinated C-fibers, which respond to noxious stimuli and transmit the information slowly as diffuse pain (Meyer, Ringkamp, Campbell & Raja, 2013). In contrast, myelinated Aδ-fibers

mediate faster and more precise pain information to the brain (Meyer et al., 2013). The brain areas, which are activated during pain perception in humans, were investigated by brain imaging studies and summarized in a meta-analysis by Apkarian, Bushnell, Treede, and Zubieta (2005) emphasizing a network rather than a single region responsible for pain processing. The main components are primary (S1) and secondary (S2) somatosensory, insular, anterior cingulate, and prefrontal cortices and thalamus (Apkarian et al., 2005). Likewise, NE in the LC and 5-HT are important neurotransmitters for the mediation of pain sensation signals in the brain (Fields, Heinricher & Mason, 1991; Tracey & Mantyh, 2007).

Besides the neuroimaging data of research on pain processing, further levels of pain perception like subjective pain sensation as well as physiological and electro-cortical responses are of great interest to understand multiple facets of various pain processing. First, subjective algesimetry comprises for instance ratings of pain intensity or unpleasantness, the assessment of pain threshold and tolerance not only in experimental settings with healthy participants but also in patients (Childs, Piva & Fritz, 2005; Göbel & Westphal, 1987; Tousignant-Laflamme, Rainville & Marchand, 2005). Second, objective algesimetry may be implemented for instance by measuring physiological and electro-cortical responses to pain. As such, electrodermal activity increases (Storm, 2008) and HR accelerates (Tousignant-Laflamme et al., 2005), whereas the attention to visual stimuli measured by event-related potentials attenuates during painful stimulation (Bromm & Lorenz, 1998; Kenntner-Mabiala & Pauli, 2005). Pain can be induced experimentally in several ways. Two of these are relevant for the current study: pressure pain and electrical pain. The former is very well suited to induce a tonic pain sensation, which can be induced for example by a device applying a defined amount of weight on the back of the phalanx of fingers using a flat-tipped stylus (Göbel & Westphal, 1987; Göbel, 1986; Kenntner-

Mabiala, Weyers & Pauli, 2007; Wieser, Gerdes, Greiner, Reicherts & Pauli, 2012). A study in monkeys by Slugg, Meyer, and Campbell (2000) suggested both, the activation of A-fibers and C-fibers due to pressure pain. Tonic pain is modulated by attention towards the painful stimulus compared to emotional stimuli in the environment (Kenntner-Mabiala et al., 2007), the differentiation of emotions in the environment, however, stays intact (Wieser et al., 2012). Interestingly, Göbel and Westphal (1987) found a lateral asymmetry only in women, demonstrating higher pain sensitivity on the fingers of the non-dominant hand vs. fingers of the dominant hand. The pain threshold is higher for male compared to female participants and slightly higher on the dominant vs. non-dominant hand (Brennum, Kjeldsen, Jensen & Staehelin Jensen, 1989).

Cutaneous phasic electric stimulation leads to excitation of local nerve fibers resulting in distinct, sharp noxious sensations (Basbaum & Jessel, 2013; Riley III, Robinson, Wise, Myers & Fillingim, 1998). Dependent on the stimulation intensity, different fibers were stimulated: highly myelinated Aα fibers have the lowest electrical resistance, and therefore are already activated by low mechanical stimulation intensities (Basbaum & Jessel, 2013). By increasing intensity, Aβ fibers and finally nociceptive Aδ fibers are activated (Scholz & Woolf, 2002), which can lead to pain sensation (Basbaum & Jessel, 2013). When the stimulation intensity is even more increased, unmyelinated C fibers are activated, which leads to extreme pain sensation (Basbaum & Jessel, 2013). As for pressure pain, electrical pain sensation can be measured by pain ratings of tolerance, intensity, and unpleasantness; the higher the electric stimulation, the shorter the tolerance and the higher intensity and unpleasantness ratings (Breimhorst et al., 2011; Rainville, Feine, Bushnell & Duncan, 1992). In addition to the peripheral marker of pain, somatosensory evoked ERPs (SEPs) can be measured as neurophysiological indicators of somatosensory processing. For instance, Kenntner-Mabiala and Pauli (2005) found increased SEP amplitudes for painful compared to non-painful electric stimuli measured at central electrode sites.

From a clinical perspective, pain perception can be altered in various ways: The motivational priming hypothesis according to Lang (1995) describes that dependent on the emotional state of a person the valence of a stimulus is perceived differently. In particular, a person in a negative emotional state displays facilitated processing of aversive stimuli and inhibited the processing of appetitive stimuli (Kenntner-Mabiala & Pauli, 2005). In this way, aversive electric stimuli were rated as less unpleasant and less intense when a positive picture was presented simultaneously (Kenntner-Mabiala, Andreatta, Wieser, Mühlberger & Pauli, 2008). In line with these results, somatosensory ERPs on following painful electric stimuli were modulated by the valence of simultaneously presented pictures (Kenntner-Mabiala et al., 2008). Besides emotions (Wieser, Gerdes, Reicherts & Pauli, 2014), on the one hand, drugs, like opioids modulate pain on a neurobiological level (for a review see Inturrisi & Jamison, 2002) and even the use of placebos (Beecher, 1955; Reicherts, Gerdes, Pauli & Wieser, 2016), and on the other hand, psychological pain modulation like emotion regulation (Jackson et al., 2012; Keefe & Williams, 1990; Kohl, Rief & Glombiewski, 2013) can change the perception of pain. Interestingly, Usichenko, Hacker, and Lotze (2017) investigated randomized clinical trials of chronic and acute pain patients and reported analgesic effects induced by auricular acupuncture, particularly by stimulating the ABVN. Transcutaneous vagus nerve stimulation exactly taps into the same mechanism of stimulating the ABVN and is therefore tested as a useful new method for pain reduction (Yuan & Silberstein, 2016a, 2016b, 2016c). According to this idea, activation of the ipsilateral NTS by vagus nerve stimulation leads to activation in limbic system, raphe nucleus, amygdala, periaqueductal gray (PAG) and hypothalamus, which modulate emotional as well as autonomic pain reactions (De Couck et al., 2017; Kirchner, Birklein, Stefan & Handwerker, 2001). Though the exact mechanism which VNS boosts during activation is still under speculation (Mauskop, 2005). NTS, nucleus raphe magnus, LC, and subcoeruleus might be involved in analgesia induced by VNS (Randich,

1992). Both, chronic and acute pain reduction by VNS have already been investigated. Single case reports by Sadler, Purdy, and Rahey (2002) and Kirchner, Birklein, Stefan, and Handwerker (2000) described a patient suffering from refractory epilepsy and additionally from migraine or headaches, respectively. Due to the epilepsy treatment, the patients got an implanted vagus nerve stimulator. Subsequently, headache symptoms mitigated in both cases (Kirchner et al., 2000; Sadler et al., 2002). More specifically, some patients with intractable migraine and cluster headaches were treated with implanted VNS and good to excellent responses were shown in two-thirds of the patients (Mauskop, 2005). However, as long as vagus nerve stimulators had to be implanted by surgery, pain research could ethically only be implemented in patients who suffered from a severe disease like epilepsy and therefore wore a VNS device (Ness, Fillingim, Randich, Backensto & Faught, 2000). Kirchner et al. (2000) found analgesic VNS effects in epilepsy patients for experimentally induced tonic but not phasic pain. Comorbidities and heterogenous medications of patients taking part in such studies made the systematic investigation of promising pain-reducing effects of VNS difficult and results speculative (Kirchner et al., 2000; Multon & Schoenen, 2005; Ness et al., 2000). Along with the investigation of tVNS, highly controlled experiments on the modulation of pain in healthy participants became possible, which allowed more general and solid conclusions on the role of vagus nerve activation for pain modulation. Busch et al. (2013) systematically investigated the effects of tVNS in healthy participants using a standardized quantitative sensory testing procedure simultaneous to either tVNS or sham stimulation in a within-subject design. Interestingly, the tVNS condition increased pain thresholds of mechanical and pressure stimulation and additionally reduced pain responses of mechanical stimulation compared to the sham condition (Busch et al., 2013). However, they could not show altered physiological responses of cardiac activity, i.e. changes in heart rate induced

by the modulation of pain (Busch et al., 2013). Nevertheless, the replication of analgesic effects by vagus nerve stimulation seems to be a promising approach to investigate the correct application and functioning of a transcutaneous vagus nerve stimulator.

As Study 2 showed, the most effective stimulation parameters and protocols for tVNS in humans are still unknown and a manipulation check for the validation of the effectiveness of the stimulation is missing. Therefore, the validation of the stimulation parameters used in Study 2 was necessary. For this purpose, the replication of tVNS results with the above-used parameters could be proof for reliable vagus nerve stimulation parameters. For this manipulation check, Study 3 investigated the modulatory effects of tVNS on pain perception, which had been investigated previously in mechanical pain, and compared it with acute electric pain. More precisely, current study aims are first to serve as manipulation check that tVNS in Study 2 stimulated the vagus nerve; second to replicate analgesic effects of tVNS during tonic pressure pain with the stimulation parameters used in Study 2; third to extend the investigation of the analgesic effects to phasic painful electric stimulation. More specifically, I hypothesized higher pain tolerance levels and lower pain intensity and unpleasantness ratings for pressure pain in participants who received tVNS compared to a sham stimulated and a control group, respectively. Additionally, I expected higher electric pain thresholds and lower pain ratings in the tVNS compared to the sham and control group after the stimulation. Although evidence so far is inconclusive (Busch et al., 2013; Genheimer et al., 2017), I decided to record HR as a physiological indicator of effective vagus nerve stimulation.

4.2 Material and methods

Within the scope of this study, Lisa-Marie Krause, Jessica Ruck and Karin Sindern wrote their Bachelor Theses under my supervision.

4.2.1 Participants

The internet platform SONA by the University of Würzburg (psywue.sona-systems.com) was used for the recruitment of participants. We tested healthy male and female participants aged between 18 and 35 years, who had no experience with vagus nerve stimulation prior to the experiment. Additional exclusion criteria were pregnancy, left-handedness, and the intake of any analgesic drugs. In total, 91 participants were invited to take part in the experiment and were randomly assigned to either the tVNS, the sham or the control group. Thirteen participants had to be excluded from the analysis. Three participants did not come to the laboratory or quit the experiment, one did not follow the instructions properly, the others were excluded due to technical problems either with administration of painful stimuli (N = 5), with physiological measurement (N = 3) or with the vagus nerve stimulator (N = 1). Out of the remaining 78 participants (age: M = 23.32 years, SD = 3.72), 27 participants (7 male) were assigned to the tVNS group, 25 participants (8 male) to the sham and 26 participants (5 male) to the control group with similar gender ratio ($X^2(2) = 1.09$, $p = .579$). Participants received either 2.5 hours course credit or 16 €.

4.2.2 Stimulus Material

The experiment was programmed with *Presentation* software (Version 15.1, Neurobehavioral Systems, Inc, Albany, USA.). Instructions for the experiment were presented on a Powerwall.

Electric stimulation. The electric stimulus was applied as described above using a Digitimer Constant Current Stimulator Model DS7A (Digitimer Ltd, Hertfordshire, England). All electric stimuli were applied with a pulse frequency of 50 Hz and lasted for 200 ms. The individual pain threshold was determined on the right arm by the same procedure explained above in the conditioning studies. The level of the individual pain threshold was increased by 30% for the stimulation used during the experiment. During pain measurement, electric stimuli were applied on the right and on the left inner forearm.

Pressure Stimulation. The electrically driven device for the application of painful pressure stimulation has been described previously (Ellermeier & Westphal, 1995). The apparatus contained a lever mechanism and an adjustable weight between 0 and 970 g resulting in pressure of 0 to 1339 kPa. For the current experiment, I used a weight of 550 g resulting in a constant pressure of approximately 763 kPa, which is in the range of previous studies (Göbel & Westphal, 1987; Göbel, 1986; Kenntner-Mabiala et al., 2007; Wieser et al., 2012). At the end of the lever, a flat-tipped stylus with a diameter of 3 mm could be lowered pressing on the phalanx of a finger. Stimulation sites comprised the middle and proximal phalanxes of the index finger, middle finger, and ring finger of the left and the right hand.

Vagus nerve stimulation. The transcutaneous vagus nerve stimulation was performed as described in Study 2. The device as well as the stimulation procedure for the determination of participants' individual stimulation threshold were the same in both experiments. Neither mean stimulation intensities of tVNS (M = 1.2 mA, SD = 0.5) and sham (M = 1.2 mA, SD = 1.0) differed ($t(50) = 0.13$, $p = .895$), nor the ratings of the stimulation (tVNS: M = 6.7 mA, SD = 0.7; sham: M = 6.5 mA, SD = 0.8; $t(50) = 0.90$, $p = .375$).

4.2.3 Measures

Questionnaires. Besides a demographic questionnaire, the ASI (German version by Alpers & Pauli, 2001; Reiss et al., 1986), the STAI (German version by Laux et al., 1981; Spielberger et al., 1970), the morningness-eveningness-Questionnaire (D-MEQ; German version by Griefahn, Künemund, Bröde & Mehnert, 2001; Horne & Ostberg, 1976), the Rosenberg Self-Esteem Scale (RSE; Rosenberg, 1965; German version by von Collani & Herzberg, 2003), the PANAS (German version by Krohne et al., 1996; Watson et al., 1988), and the pain sensitivity questionnaire (PSQ; Ruscheweyh, Marziniak, Stumpenhorst, Reinholz & Knecht, 2009) were assessed. The latter describes participants' general pain perception by asking for their pain sensitivity in 17 daily situations e.g. *Imagine you bump your shin badly on a hard edge, for example, on the edge of a glass coffee table. How painful would that be for you?* The rating scale ranges from 0 (= *not at all painful*) until 10 (= *most severe pain imaginable*). Please note that PANAS (Krohne et al., 1996) and STAI state (Laux et al., 1981) were assessed pre and post experimental pain procedure. After the experiment, a self-developed questionnaire similar to the questionnaire in Study 2 about participants' subjective experience with the vagus nerve stimulation asked for comfort as well as the personal opinion and side effects. Hence, participants rated their conviction of the stimulation efficacy (0 = not convinced, 10 = very convinced), the operability of the stimulation (0 = stimulation did not work, 10 = stimulation worked well), the valence of the stimulation (0 = unpleasant, 10 = pleasant), their estimation of VNS for the clinical use (0 = I can't imagine a clinical use of the stimulation, 10 = I can very well imagine the stimulation for clinical use), and whether they felt any side effects of the stimulation.

Ratings. For the electric pain ratings, we used the same scale as for the determination of the electric pain threshold, namely on an 11-point Likert scale (0 = *no sensation at all*, 4 = *just noticeable pain*, 10 = *unbearable pain*). Numeric rating scales of 11 steps were used

for ratings of pressure pain intensity and pain unpleasantness (Villemure, Slotnick & Bushnell, 2003). Therefore, participants were asked '*How intense was the painful stimulus?*' and gave their answer on a scale ranging from 0 (= *not at all painful*) to 10 (= *most severe pain imaginable*). In parallel, for pain unpleasantness ratings, participants were asked '*How unpleasant was the painful stimulus?*' and rated on a scale from 0 (= *not at all unpleasant*) to 10 (= *extremely unpleasant*). Additionally, pressure pain tolerance was assessed by the time participants could tolerate the pressure pain. They were explicitly asked to press a button on a keyboard to lift the pressure stylus when they could not tolerate the pain anymore.

HR. The electrocardiogram for recording the heart rate was measured by three single-use adhesive Ag/AgCl foam electrodes of 55 mm diameter by Swaromed. One electrode was placed on the right clavicle, the second on the left lower costal arch and the third on the back above the left hip. On those locations, we cleaned participants' skin with alcohol and attached the electrodes. Data were recorded continuously by the Vision Recorder software (version 1.21, Brain Products Inc., Munich, Germany) with a sampling rate of 1000 Hz and an online Notch filter of 50 Hz.

4.2.4 Procedure and Design

After arriving in the laboratory, participants read the information sheet and signed the informed consent. In case of any question, participants were encouraged to ask the experimenter for further information. Subsequently, participants filled in the questionnaires. ECG electrodes, as well as the electrode for the electric stimuli, were attached. Depending on the participant's group assignment (see Figure 5), the vagus nerve stimulator was either attached to the cymba conchae (VNS and control condition) or the helix (sham condition).

The experimental procedure for each experimental group is depicted in Figure 11. At the beginning of the experiment for the pre-stimulation measures, the participant's individual pain threshold for electric stimuli was determined on the right arm by the procedure described in Study 1. After increasing the threshold intensity by 30%, three electric stimuli were applied to each forearm, which was always followed by a verbal pain rating on the pain scale for electric stimuli (see Figure 11 for the exact timing). Subsequently, six trials of pressure pain were performed. Therefore, the flat-tipped stylus was lowered on the middle phalanx of index, middle, or ring finger of both hands. After 25 s of pressure, verbal pain intensity, and after 30 s pain unpleasantness ratings were requested by the respective questions appearing on the powerwall and the answers were noted by the experimenter. Within this trial, five seconds later pain tolerance was measured by asking the participant to lift the lever whenever they do not tolerate the pain anymore. A trial was terminated after a maximum of 2 min. After the pre-measures, participants assigned to the VNS or sham group underwent the work-up for the individually optimal stimulation intensity. Subsequently, a 20 min stimulation of 30 s on/off cycles was applied while participants were sitting calmly in the laboratory. For a detailed description of the procedure see Study 2. After VNS or sham stimulation, the electric pain threshold was assessed a second time. Subsequently, twelve painful electric stimuli were applied – 6 on each forearm – by interchanging the stimulation intensity from the first and the second pain threshold (pre and post). During the application, either vagus nerve, helix or no ear stimulation was given (for the exact timing see Figure 11). The rating was the same as in the pre-VNS procedure. Similarly, the pressure stimuli were again applied, pain intensity and unpleasantness ratings were assessed, pain tolerance was measured and, depending on the participant's group, ear stimulation was given. In addition to the previously used pressure stimulation sites (post1), six additional sites on

the proximal phalanx next to the fingernail of the same fingers were added (post2). In the end, participants filled in the PANAS (Krohne et al., 1996), STAI state (Laux et al., 1981) and the VNS stimulation questionnaire, and the experimenter debriefed participants in the sham and control group.

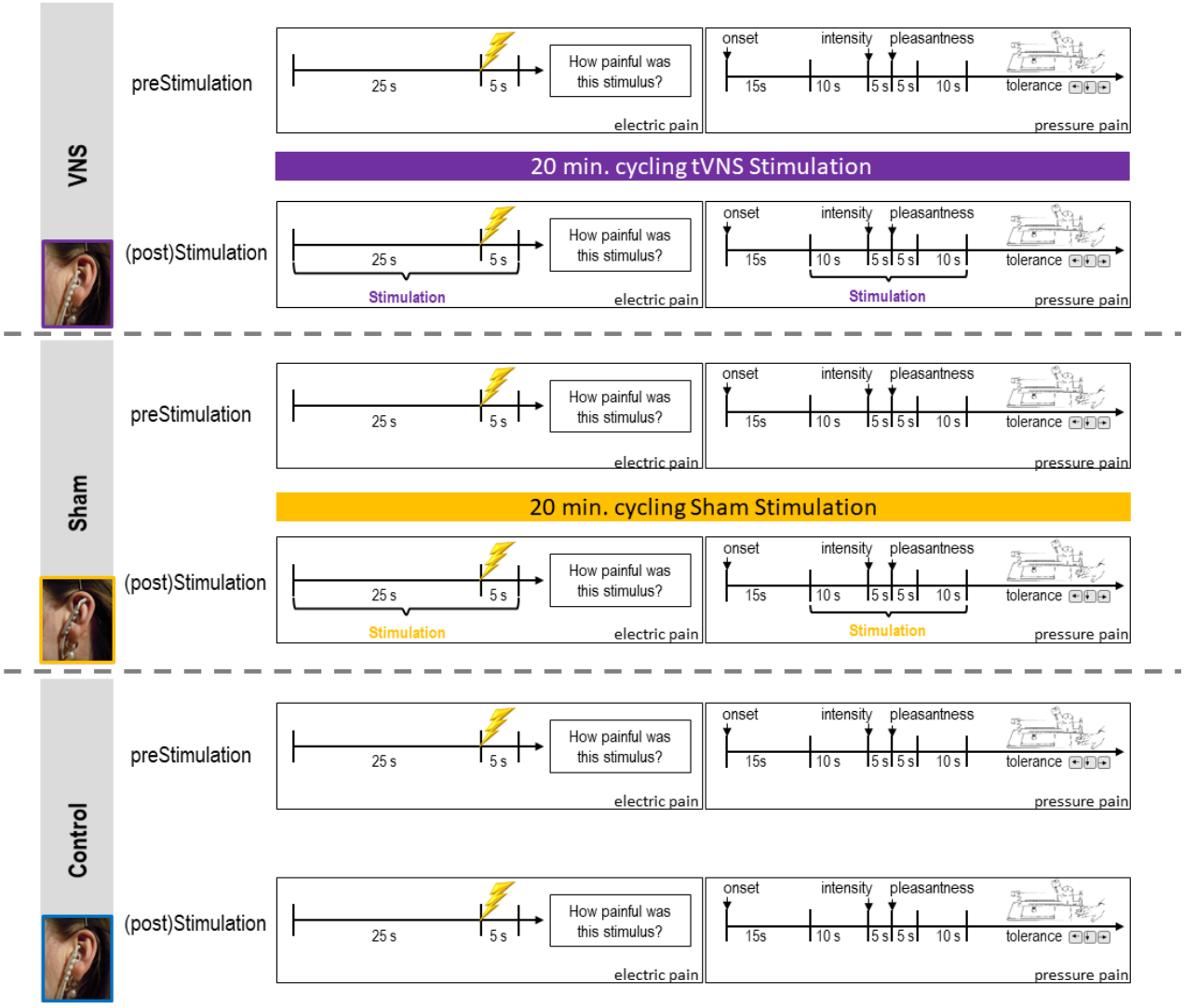

Figure 11: Experimental procedure of Study 3.

Depicted are the different protocols separately for each group (tVNS: upper panel, sham: middle panel, control: lower panel). All groups started with the electric pain stimulation followed by pressure pain. After 20 min of tVNS, sham or without stimulation (control) the procedure was repeated with the respective simultaneous ear stimulation. Please note that the pressure pain procedure post stimulation included the same six stimulation sites on the middle phalanxes of the fingers, which have been used in the pre stimulation test (post1), and six additional stimulation sites on the proximal phalanxes of the fingers (post2).

4.2.5 Data Recordings and Data Reduction

HR. ECG data were preprocessed with the program Brain Vision Analyzer (Version 2.0, Brain Products GmbH, Munich, Germany). After the data filtering with a low pass filter of 30 Hz, segments of 30 s were cut. In the case of electric pain stimulation trials, a segment consisted of 25 s before and 5 s after the electric stimulus. For the pressure pain, 30 s segments were cut starting when the lever lowered on the finger. Semi-automatic R-peak detection, which is implemented in the software, was used and manually corrected. Afterward, the heart rate was readout (see Study 2). The averaged HR values of intervals of 5 s were exported and analyzed for each condition.

4.2.6 Statistical Analyses

The state questionnaires were analyzed with a mixed ANOVA with the within-subject factor time (pre, post) and the between-subjects factor group (tVNS, sham, control). For the trait questionnaires as well as for the stimulation conditions, univariate ANOVAs were calculated containing the between-subjects factor group (tVNS, sham, control). The number of side effects of the stimulation was compared by a χ^2-test.

The electric pain thresholds were compared by a mixed ANOVA with the within-subjects factor time (pre, post) and the between-subjects factor group (tVNS, sham, control). The pain ratings for electric as well as for pressure pain were analyzed by repeated-measure ANOVAs with the within-subject factors phase (pre, post1, post2), side (right, left) and the between-subjects factor group (tVNS, sham, control). The analyses for the heart rate additionally included the within-subject factor time (T1, T2, T3, T4, T5, T6), which were calculated by the mean HR of 5 s intervals, i.e. 0-5 (T1), 5-10 (T2), etc.

For the analysis of pressure pain tolerance, the time of participants' pain tolerance was assessed. Participants who tolerated the pressure pain for more than 2 min were excluded from analyses. Significant main effects and interactions were dissolved post-hoc by

dependent and independent samples *t*-tests, respectively. In case the assumption of sphericity was violated, Greenhouse-Geisser corrections were applied and Greenhouse-Geisser Epsilon (*GG-ε*) reported. For all statistical tests, the α-niveau was set at 0.05.

4.3 Results

4.3.1 Questionnaires

In all groups, neither the positive affect nor the negative affect (Krohne et al., 1996) changed over time (all *p*s > .090). Interestingly despite the anticipation of a pain including experiment, the state anxiety (Laux et al., 1981) at the beginning of the experiment was similar to the end of the experiment independent of the factor group (all *p*s > .075). An overview of the group comparisons of the trait questionnaires is depicted in Table 6.

The scores of the ASI (German Version by Alpers & Pauli, 2001; Reiss et al., 1986), of the D-MEQ (German version by Griefahn et al., 2001; Horne & Ostberg, 1976) as well as the PSQ (Ruscheweyh et al., 2009) did not differ between groups (all *p*s > .530). However, scores on trait anxiety (German version by Laux et al., 1981; Spielberger et al., 1970) statistically differed between groups ($F(2,77) = 4.08$, $p = .021$, $\eta_p^2 = .099$). Post-hoc *t*-tests revealed significantly higher trait anxiety for participants in the tVNS ($t(51) = 2.50$, $p = .016$) and sham ($t(49) = 2.84$, $p = .007$) group compared to control participants. The tVNS and sham group had similar trait anxiety scores ($t(50) = 0.06$, $p = .957$). Similarly, the RSE Scale (Rosenberg, 1965; German version by von Collani & Herzberg, 2003) revealed differences between groups ($F(2,77) = 7.76$, $p = .001$, $\eta_p^2 = .207$). Though the tVNS group and controls did not differ in RSE scores indicated by post-hoc *t*-tests ($t(51) = 1.76$, $p = .084$), the sham group had lower RSE scores than tVNS group ($t(50) = 2.21$, $p = .032$) and controls ($t(49) = 3.77$, $p = .001$).

Table 6: Mean trait questionnaire scores of Study 3 compared between groups.

	tVNS	sham	control	statistics
STAI trait (SD)	39.78 (10.11)	39.92 (8.50)	34.08 (6.03)	$F(2,77) = 4.08$; $p = .021$
ASI (SD)	16.15 (7.10)	18.12 (7.77)	16.08 (7.12)	$F(2,77) = 0.64$; $p = .531$
RSE (SD)	38.81 (4.46)	35.60 (5.96)	40.92 (3.28)	$F(2,77) = 7.76$; $p = .001$
DMEQ (SD)	50.22 (11.13)	50.24 (8.89)	50.62 (9.01)	$F(2,77) = 0.01$; $p = .987$
PSQ (SD)	3.92 (1.01)	4.21 (1.26)	4.23 (1.42)	$F(2,77) = 0.52$; $p = .596$

In order to control for the influence of trait anxiety and self-esteem, I calculated additional Analysis of Covariance (ANCOVAs) with the respective variable as a covariate for all analyses. In case of significant interactions of a dependent variable with the questionnaire, the ANCOVA and correlations were reported.

4.3.2 Stimulation conditions

For the tVNS as well as for the sham group, participants' stimulation intensity was determined according to the procedure described above (Study 2). Neither the stimulation intensity nor the subjective estimation of participants' perceived stimulation differed between groups (all *p*s > .374; see Table 7).

Univariate ANOVAs revealed similar conviction of the stimulation efficacy as well as similar participants' perception of the clinical use of the stimulation method (*p*s > .073, see Table 7). However, groups differed regarding their estimation of the valence ($F(2,77) = 5.41$, $p = .006$, $\eta_p^2 = .126$) and the operability of the stimulation ($F(2,77) = 12.82$, $p < .001$, $\eta_p^2 = .255$). Valence of the stimulation was rated as more pleasant in the control vs. tVNS group ($t(51) = 3.29$, $p = .002$), but similar pleasant in control and sham ($t(49) = 1.38$, $p = .173$) as well as tVNS and sham group ($t(50) = 1.85$, $p = .070$). Both groups with active

stimulation, i.e. tVNS and sham, reported better operability in comparison to the control group ($t(51) = 4.25$, $p < .001$ and $t(49) = 4.16$, $p < .001$, respectively).

Table 7: Comparison of mean ratings of the stimulation between groups in Study 3.

	tVNS	sham	control	statistics
Stim. intensity (SD)	1.20 (0.49)	1.23 (0.96)	-	$F(1,51) = 0.02$, $p = .895$
Stim. rating (SD)	6.67 (0.73)	6.48 (0.77)	-	$F(1,51) = 0.80$, $p = .375$
Conviction (SD)	5.85 (1.81)	5.52 (2.04)	4.54 (2.72)	$F(2,77) = 2.48$, $p = .090$
Operability (SD)	6.85 (2.54)	6.76 (2.35)	3.54 (3.11)	$F(2,77) = 12.82$, $p < .001$
Valence (SD)	4.70 (2.09)	5.80 (2.18)	5.73 (3.08)	$F(2,77) = 5.41$, $p = .006$
Clinical use (SD)	7.00 (2.17)	7.24 (2.19)	6.60 (2.48)	$F(2,77) = 2.69$, $p = .074$
Side effects (N)	8	3	3	$\chi^2(2) = 3.83$, $p = .148$

Additionally, a similar number of participants reported side effects of the ear stimulation ($\chi^2(2) = 3.83$, $p = .148$) at the end of the experiment. Prickling, a sensation of heat, slight itching, goosebumps, pressure in the ear, tingling, grumbling stomach, fatigue, and minimal pain were amongst the side effects that were reported by the participants.

4.3.3 Electric pain

Threshold. The determination of participants' individual pain threshold was assessed prior to the experiment and directly after the 20 min stimulation phase.

Table 8: Comparison of mean electric pain threshold between groups in Study 3.

	tVNS	sham	control	statistics (Time x Group)
Stim. intensity pre (SD)	2.27 (0.23)	1.80 (0.24)	1.90 (0.24)	$F(2,75) = 2.16$, $p = .122$
Stim. intensity post (SD)	1.95 (0.24)	1.72 (0.25)	1.99 (0.25)	
Stim. rating pre (SD)	5.52 (0.12)	5.56 (0.12)	5.54 (0.12)	$F(2,75) = 1.07$, $p = .349$
Stim. rating post (SD)	5.70 (0.14)	5.52 (0.15)	5.42 (0.15)	

The ANOVA for the determined threshold intensity revealed neither significant main effects of time, nor group, nor an interaction of Time x Group (all *p*s > .121) meaning that neither the time nor the kind of stimulation had an impact on participants' electric pain threshold. As expected, the ratings of the pain were similar in the pre and post pain threshold determination for all groups (all *p*s > .348), since the rating scale was the reference for the intensity of the electric stimulation.[2]

Electric pain ratings. During the post measures, the intensity of the electric stimulus was alternated between the threshold determined pre and post. However, due to the similar stimulation intensity and subjective pain ratings reported above, I averaged the pain ratings of 3 subsequently applied electric stimuli. Then, an ANOVA with the within-subject factor time (pre, post1, post2), side (left, right), and the between-subjects factor group (VNS, sham, control) was calculated. The ANOVA[3] revealed a main effect of time

[2] By controlling for RSE, the ANCOVA for ratings of the electric pain threshold revealed an interaction of Time x RSE ($F(1,74) = 4.07$, $p = .047$, $\eta_p^2 = .052$), which only indicated a marginally positive correlation ($r(78) = .198$, $p = .082$).

[3] Please note that 3 additional participants (1 from tVNS and 2 from control group) had to be excluded from this calculation due to technical problems with the application of the electric stimuli.

($F(2,144) = 17.09$, $GG\text{-}\varepsilon = .785$, $p < .001$, $\eta_p^2 = .192$), a significant interaction of Time x Side ($F(2,144) = 9.08$, $GG\text{-}\varepsilon = .785$, $p = .001$, $\eta_p^2 = .112$), and marginally significant interactions of Time x Group ($F(4,144) = 2.47$, $GG\text{-}\varepsilon = .785$, $p = .063$, $\eta_p^2 = .064$, see Figure 12).

Noteworthy, neither the main effects of group nor side nor the interactions of Side x Group nor of Time x Side x Group reached significance (all *p*s > .090). Post-hoc *t*-tests for the Time x Side interaction revealed significantly higher electric pain ratings on the right

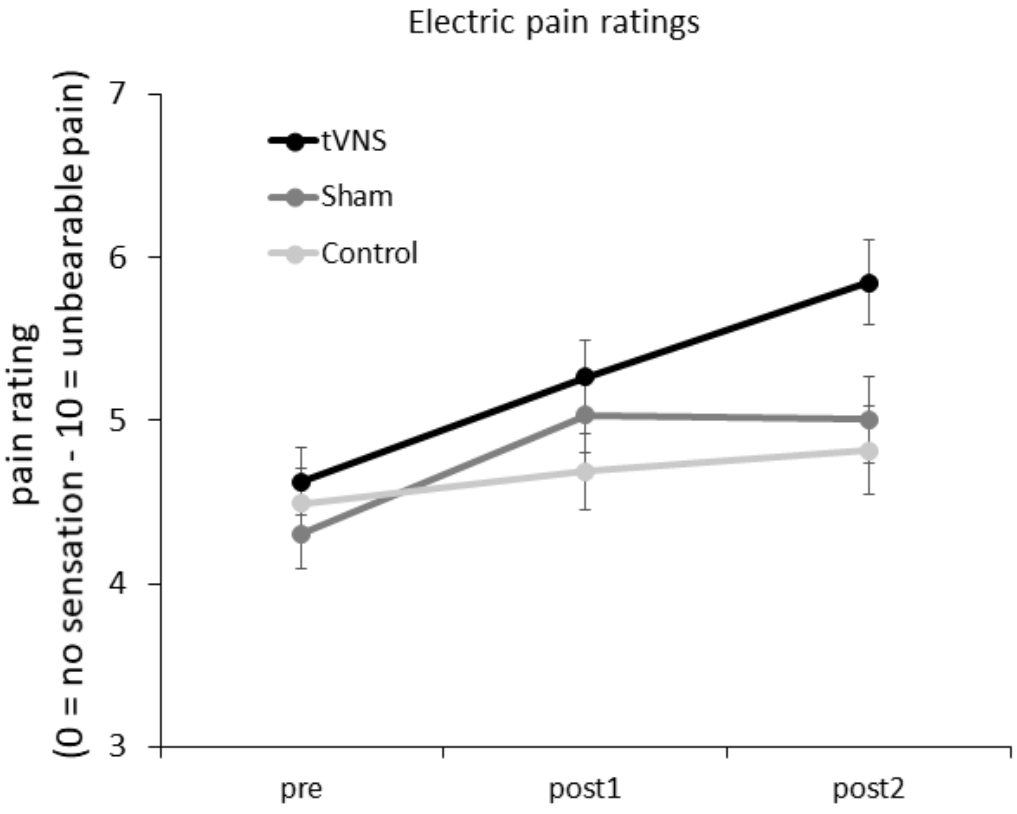

Figure 12: Electric pain ratings in Study 3 separately for each group.

Depicted are the mean pain ratings averaged across the 6 electric stimuli before stimulation (pre), across the 6 first electric stimuli (post1) and after the 6 last electric stimuli (post2) on the left and right arm after the interval stimulation. In black, ratings of the tVNS group, in dark gray ratings of the sham group and in light gray ratings of the control group are shown. Standard errors are depicted.

compared to the left arm before any intervening stimulation ($t(74) = 2.84$, $p = .006$), but similar ratings for the right and the left arm at the first and second test phase after the stimulation block and concurrently with the respective stimulation (all *p*s > .115). In total,

the stimulation did not seem to have any influence on participants' subjective electric pain perception.[4]

HR: The ANOVA for the HR[5] in anticipation and after the mildly painful electric stimulus revealed a main effect of time ($F(5,295) = 51.82$, $GG\text{-}\varepsilon = .562$, $p < .001$, $\eta_p^2 = .468$) and indicated changing HR over the recorded period of 30 s. In particular, findings

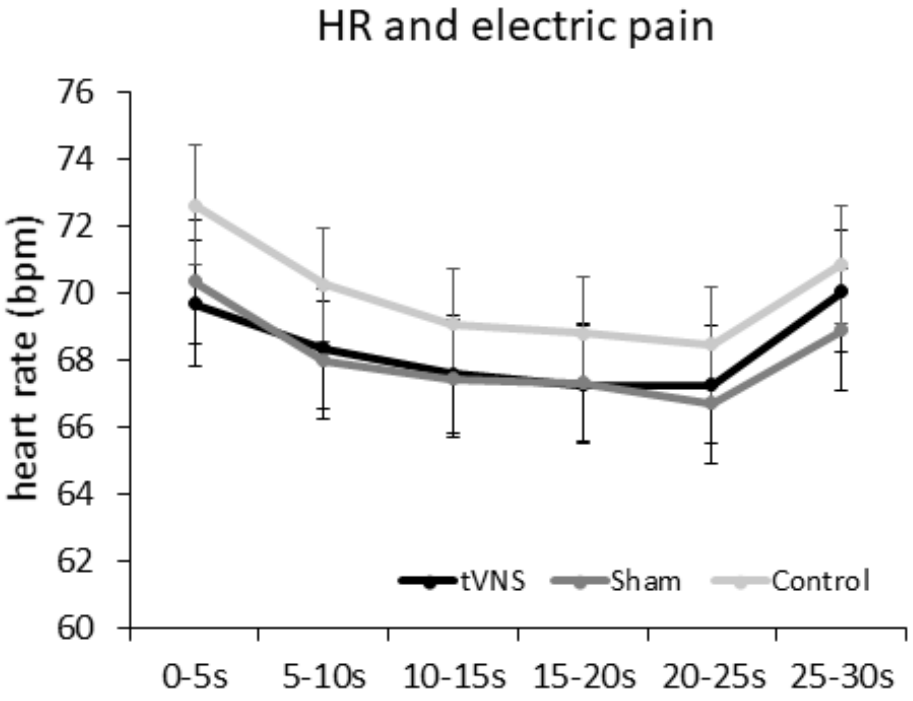

Figure 13: Heart rate and electric pain in Study 3.

The electric stimulus was applied after 25 s. Depicted is the HR separately for each group: tVNS in black, sham in dark gray and control in light gray with standard errors (SE).

[4] I controlled for the different scores of the STAI trait questionnaire between groups by calculating an ANCOVA with STAI trait as a covariate and found a significant interaction of Side x STAI trait ($F(1,71) = 4.26$, $p = .043$, $\eta_p^2 = .057$). Following this up, the correlation between differential ratings (left vs. right side) and STAI trait revealed a significant positive correlation ($r(75) = .253$, $p = .029$) meaning the more anxious participants were the bigger was the difference between their pain ratings for electric stimulation on the right and left arm. One reason for this unexpected effect might be related to the determination of the threshold, in fact, this was conducted only on the right arm. Changing the electrode to the left side could have induced more uncertainty about the pain sensation and such uncertainty modulated the pain ratings especially those high anxious participants.

[5] Due to technical problems during physiological measurement at any point of time during the experiment, 12 additional participants had to be excluded from HR analyses (7 from tVNS, 3 from sham, 4 from control) resulting in tVNS: N = 20; sham: N = 20; control: N = 22.

demonstrated heart rate deceleration during the first 25 s and an acceleration after the application of the electric stimulus (t1 vs. t2: $t(61) = 9.85$, $p < .001$; t2 vs. t3: $t(61) = 4.57$, $p < .001$; t3 vs. t4: $t(61) = 1.84$, $p = .070$; t4 vs. t5: $t(61) = 2.80$, $p = .007$; t5 vs. t6: $t(61) = 7.80$, $p < .001$; see Figure 13). The main effect of phase ($F(2,118) = 35.88$, $p < .001$, $\eta_p^2 = .378$) revealed higher HR during electric pain stimulation in the pre compared to the post phases (post1: $t(61) = 6.69$, $p < .001$, post2: $t(61) = 5.64$, $p < .001$) and higher HR in post2 compared to post1 ($t(61) = 2.33$, $p = .023$). A main effect of side ($F(1,59) = 6.24$, $p = .015$, $\eta_p^2 = .096$) indicated higher HR when the electric stimulus was applied on the left compared to the right arm. Importantly, neither effect of group nor its interactions reached significant levels (all *p*s > .145) assuming no influence of tVNS stimulation on heart rate.

4.3.4 Pressure pain

Intensity ratings. Comparing the pain intensity ratings pre stimulation and directly after the stimulation block (post1) and later (post2), a main effect of phase ($F(2,150) = 36.65$, $p < .001$, $\eta_p^2 = .328$) revealed similar ratings for pre compared to post1 ($t(77) = 0.98$, $p = .332$), but higher pain intensity during post2 compared to pre ($t(77) = 6.39$, $p < .001$) and post1 ($t(77) = 7.91$, $p < .001$). Neither main effects of side, nor group, nor its interactions reached significance (all *p*s > .059).[6]

Unpleasantness ratings. The unpleasantness of the stimulation was affected by the stimulation side ($F(1,75) = 3.95$, $p = .050$, $\eta_p^2 = .050$) indicating higher pain unpleasantness, when the stimulation was applied to a finger of the left versus the right

[6] In parallel to the electric pain stimulation, I have calculated ANCOVAs considering the STAI trait as a covariate. These returned a significant Phase x STAI trait interaction ($F(2,148) = 4.56$, $GG\text{-}\varepsilon = .923$, $p = .014$, $\eta_p^2 = .058$). Then, I calculated difference scores between post1 and pre, post2 and pre, and post2 and post1. Post-hoc correlations revealed a significant negative correlation between STAI trait and the pre/post1 difference scores ($r(78) = -.413$, $p < .001$) suggesting the more anxious participants are, the higher they rated the intensity of the mechanic pain in the pre block related to the post1 block.

hand. Additionally, a main effect of phase ($F(2,150) = 46.00$, $p < .001$, $\eta_p^2 = .380$) showed higher unpleasantness in the first block of pressure stimulation procedure (pre) compared to the second phase (post1; $t(77) = 2.15$, $p = .035$) indicating a potential habituation effect. Interestingly, the third phase (post2) revealed higher unpleasantness compared to pre ($t(77) = 6.62$, $p < .001$) and post1 ($t(77) = 9.07$, $p < .001$). However, neither a main effect of group nor any interaction was revealed (all ps > .320).

Tolerance[7]. The ANOVA for the pressure pain tolerance comparing pre, post1 and post2 stimulation revealed a main effect of phase ($F(2,128) = 24.17$, $p < .001$, $\eta_p^2 = .274$) indicating similar tolerance in pre and post1 ($t(73) = 1.95$, $p = .056$), but significantly

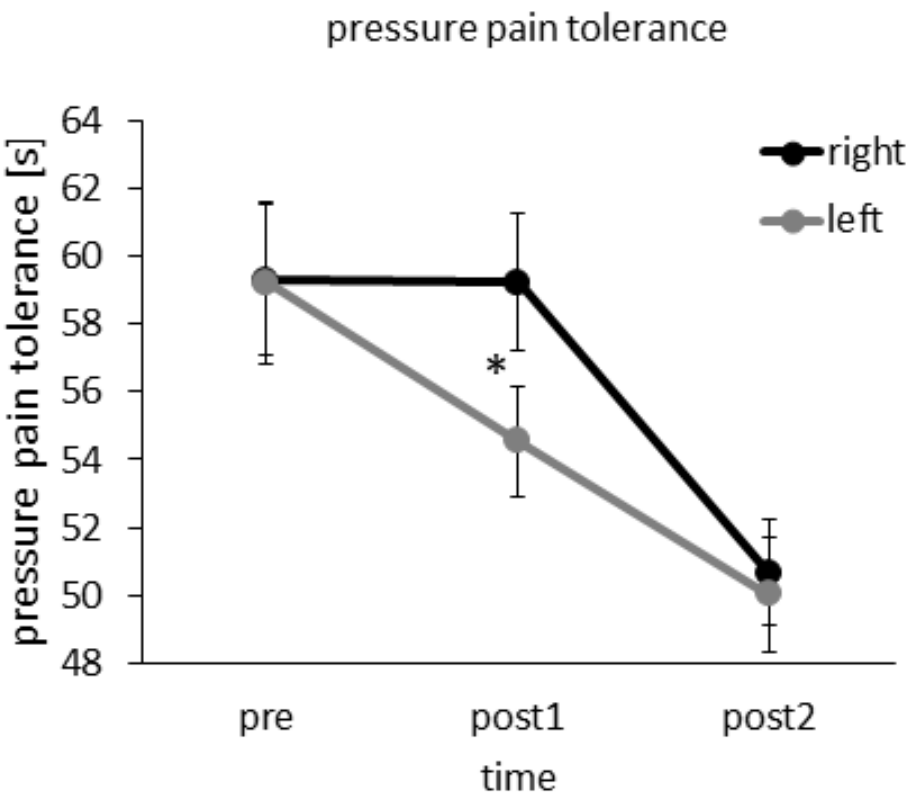

Figure 14: Pressure pain tolerance in Study 3.

Depicted is the average time in s that participants tolerated the pressure pain stimulus pre stimulation, after interval stimulation 1 (post1) and after interval stimulation 2 (post2), and the respective standard errors (SE).

[7] Please note that 11 additional participants (2 = tVNS, 6 = sham, 3 = control) had to be excluded from the pain tolerance analyses, because they tolerated the pain for more than 2 min in all three trials of one condition, resulting in tVNS: N = 25; sham: N = 19; control: N = 23.

lower pain tolerance in post2 compared to pre ($t(73) = 6.26$, $p < .001$) and post1 ($t(73) = 4.48$, $p < .001$). The significant interaction of Phase x Side ($F(2,128) = 4.73$, $p = .010$, $\eta_p^2 = .069$; see Figure 14) indicated higher pain tolerance on the right compared to the left fingers in post1 ($t(70) = 2.52$, $p = .014$), but not in pre or post2 ($ps > .384$).

HR. The ANOVA[8] for HR during pressure pain revealed main effects of time ($F(5,295) = 42.36$, $GG\text{-}\varepsilon = .432$, $p < .001$, $\eta_p^2 = .418$), phase ($F(2,118) = 5.01$, $GG\text{-}\varepsilon = .677$, $p = .019$, $\eta_p^2 = .078$), and side ($F(1,59) = 6.87$, $p = .011$, $\eta_p^2 = .104$). Moreover, the interactions of Time x Phase ($F(10,290) = 2.36$, $GG\text{-}\varepsilon = .528$, $p = .037$, $\eta_p^2 = .039$), Time x Side ($F(5,295) = 6.35$, $GG\text{-}\varepsilon = .641$, $p < .001$, $\eta_p^2 = .097$), and Time x Phase x Side ($F(10,590) = 2.44$, $GG\text{-}\varepsilon = .708$, $p = .018$, $\eta_p^2 = .040$; see Figure 15) reached significance. It is important to be aware that in case of tVNS or sham group, the ear stimulation started 15 s after the onset of the pressure stimulus, i.e. after T3.

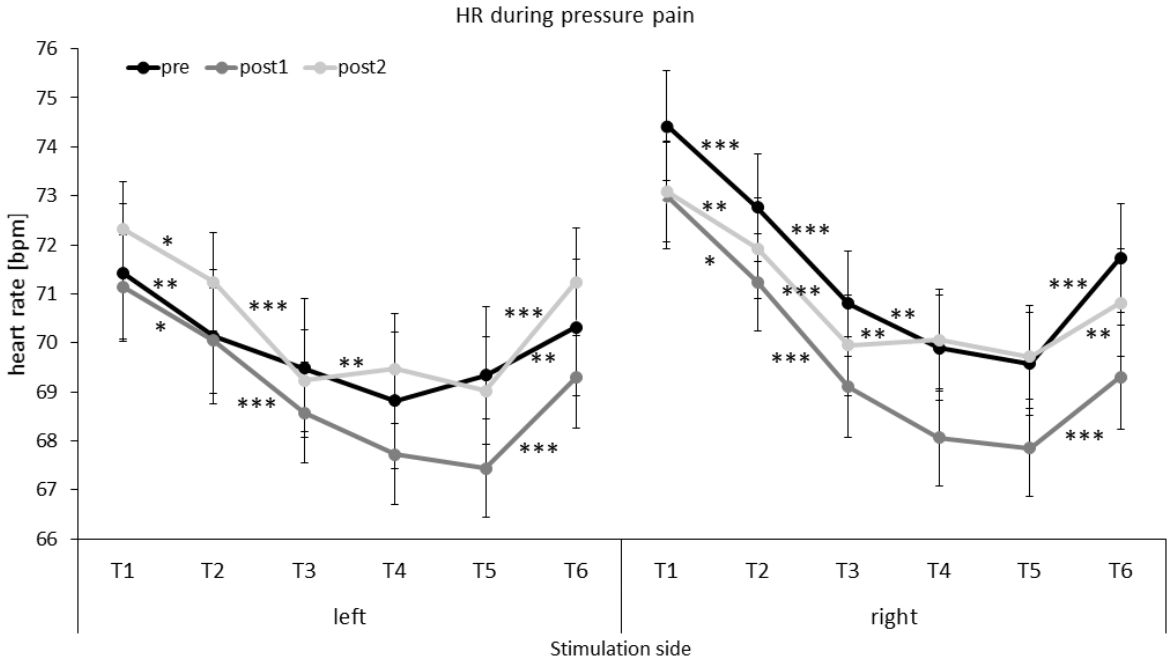

Figure 15: Heart rate during pressure pain in Study 3.

The pressure started at 0 s. The respective ear stimulation started 15 s later. Depicted are HR means and standard errors (SE) for 0-5 s (T1), 5-10 s (T2), 10-15 s (T3), 15-20 s (T4), 20-25 s (T5), 25-30 s (T6) for the left fingers (left panel) and right fingers (right panel), separately for the three phases (pre, post1, and post2). *: $p < .05$; **: $p < .01$; ***: $p < .001$

[8] Please note that the same participants were used for the HR analyses of electric pain stimulation and pressure pain. Therefore, the analysis included 62 participants: tVNS: N = 20; sham: N = 20; control: N = 22.

For the comparison of the HR without and with stimulation, I was interested in the course of HR during the 30 s of HR recording and resolved the three-way interaction by comparing adjacent time intervals with each other for all phases separately for left and right fingers. Post-hoc *t*-tests are depicted in Table 9 and show HR deceleration in the first time intervals, and HR acceleration from T5 to T6 in all conditions.

Table 9: Course of HR changes across 30 s separated for phases and side of Study 3.

	left					right				
	T1 vs. T2	T2 vs T3	T3 vs. T4	T4 vs. T5	T5 vs. T6	T1 vs. T2	T2 vs T3	T3 vs. T4	T4 vs. T5	T5 vs. T6
pre	*t*(61) = 3.25; **	*t*(61) = 1.96	*t*(61) = 1.76	*t*(61) = 1.57	*t*(61) = 3.12; **	*t*(61) = 3.93; ***	*t*(61) = 7.19; ***	*t*(61) = 2.72; **	*t*(61) = 1.16	*t*(61) = 7.51; ***
post1	*t*(61) = 2.12; *	*t*(61) = 4.30; ***	*t*(61) = 3.25; **	*t*(61) = 0.76;	*t*(61) = 5.29; ***	*t*(61) = 3.22; **	*t*(61) = 5.24; ***	*t*(61) = 2.85; **	*t*(61) = 0.68;	*t*(61) = 3.41; **
post2	*t*(61) = 2.02; *	*t*(61) = 5.62; ***	*t*(61) = 0.51;	*t*(61) = 1.55;	*t*(61) = 6.52; ***	*t*(61) = 2.43; *	*t*(61) = 4.91; ***	*t*(61) = 0.24;	*t*(61) = 1.22;	*t*(61) = 3.91; ***

*: $p < .05$; **: $p < .01$; ***: $p < .001$

Importantly, no further test including the factor group became statistically significant (all *ps* > .093), which suggests no or only small influence on tVNS stimulation on HR during pressure pain.

4.3.5 Exploratory Analyses

Valence of stimulation and pain perception. Participants in the tVNS group reported a slightly lower valence of the ear stimulation compared to both control groups. As more negative valence of a distracting stimulus could lead to higher pain ratings, I tested this exploratory. Therefore, I correlated valence ratings with pain ratings on electric stimulation and pressure pain ratings for post1 and post2, respectively. However, subjectively perceived valence of the stimulation was not related to electric pain ratings,

neither in post1 ($r(76) = .04$, $p = .715$) nor in post2 ($r(76) = .00$, $p = .974$). Similarly, correlations with pressure pain unpleasantness as well as pain intensity did not significantly correlate with the valence of the stimulation of the ratings (all ps > .505).

HR changes due to stimulation. In pressure pain, ear stimulation has begun 15 s after the pressure stylus was set on the finger. To test HR changes under pressure pain more specifically between groups, I averaged HR for all pressure pain trials on the middle phalanxes of the fingers, i.e. post1, for the 15 s before stimulation and 15 s synchronized with stimulation, respectively. Then, I compared HR without and with ear stimulation with a dependent sample *t*-test separately for each group. Interestingly, I found HR deceleration in all three groups during the time interval 15-30 s compared the time interval of 0-15 s after pressure pain onset (tVNS: $t(19) = 5.14$, $p < .001$; sham: $t(19) = 4.56$, $p < .001$; control: $t(21) = 2.44$, $p = .024$). However, no group differences were revealed.

4.4 Discussion

In the current study, my goal was to replicate the analgesic effect of tVNS on tonic pain and to extend research on acute pain using the stimulation parameters and the same control groups as in the previous experiment (Study 2). Before stimulation, I performed a phase of baseline pain measurements without ear stimulation, added a block of 20 min interval ear stimulation and afterward compared baseline to the same measurements with simultaneous ear stimulation. Unexpectedly, subjective pain ratings were neither reduced by tVNS during tonic pain, i.e. pressure pain, nor during acute pain, i.e. electric pain. More specifically, pain intensity, unpleasantness, and tolerance levels of pressure pain did not differ in tVNS, sham, and control group. Additionally, pain threshold, as well as pain ratings of painful electric stimuli, were similar across all groups. Therefore,

subjective pain reports do not allow any conclusions on an analgesic effect induced by tVNS.

Moreover, I assessed HR assuming relative deceleration in tVNS vs. sham and control group due to parasympathetic innervations of the vagus nerve to the heart (Levy, 1971). However, HR changed between groups, neither in pressure pain nor in electric pain in association with the painful stimulus after 25 s. This is in line with many studies (Busch et al., 2013; Fischer et al., 2018; Genheimer et al., 2017; Ventura-Bort et al., 2018), which did not find any changes in HR and therefore in the autonomic nervous system caused by left cymba conchae stimulation. However, a reliable manipulation check of effective stimulation parameters of the tVNS device is still necessary.

Notably, the tVNS and the sham group reported higher trait anxiety compared to the control group, which also did not seem to augment differences in pain perception between groups. The stimulation elicited higher unpleasantness in tVNS compared to sham and control group participants, which could be due to the site of stimulation. Whereas the cymba conchae stimulation for the tVNS group is located very centrally on the ear, helix stimulation for the sham group took place more distal. Importantly, the control group, which did not receive any stimulation at all, reported less operability compared to both stimulated groups. On the one hand, the additional control group improved the experimental control of the stimulation per se, on the other hand, the context in which the control group received the pain stimulation was changed by the tingling sensation at the ear and therefore might lack the trust in the operability of the stimulation device. Consequently, future studies should investigate, whether the implementation of a control group without any stimulation or a sham group with similar contextual information of tingling at the ear significantly increases the study value. Due to the similar stimulation sensation, my studies emphasized the sham group as the best control for tVNS.

Though the exact mechanisms of VNS or tVNS on pain perception are unknown, the neuronal mechanisms suggested that tVNS activates the NTS in the brain and subsequently further limbic brain structures including LC, raphe nucleus, amygdala, PAG, and hypothalamus, which modulate emotional and pain responses (De Couck et al., 2017; Kirchner et al., 2001; Randich, 1992; Yuan & Silberstein, 2016a, 2016b, 2016c). Similar to current results, Kirchner et al. (2000) found no analgesic modulation of acute pain (Kohllöffel, Koltzenburg & Handwerker, 1991) by VNS speculating that pain intensity was mainly modulated by peripheral nociceptive C fibers rather than additional central nociceptive processing which is assumed in tonic pain (Kirchner et al., 2000; Koltzenburg, Torebjörk & Wahren, 1994). Recently, Janner, Klausenitz, Gürtler, Hahnenkamp, and Usichenko (2018) reported a reduction of pain by tVNS in men using painful heat stimuli. In an elegant within-group study design, they applied no stimulation, sham, placebo and verum tVNS in four sessions which were at least 48 h apart from each other. These results emphasize the potentially hypoanalgesic effects also in acute pain. However, a closer look into those data revealed such an effect not continuously across all pain stimuli but only in response to a single heat stimulus only in male participants (Janner et al., 2018). Those gender differences were elaborated by a study demonstrating the relationship between increased parasympathetic activity resulting in more effective pain modulation only in males (Nahman-Averbuch et al., 2016). Furthermore, they found pain reduction in all interventions compared to the no intervention condition, which suggests that any additional stimulus which attracts attention reduces pain (Janner et al., 2018). Though this evidence (Janner et al., 2018) suggested specific effects of gender both on tVNS and pain perception, a proper analysis was not possible in the current sample, which only consisted of 20 males (tVNS: N = 7; sham: N = 5; control: N = 8) in total. Since the present study was not designed to answer gender-related questions, the missing power,

unfortunately, hampers further conclusions here. Further systematic interventions of these equivocal data set are urgently required for the application of tVNS in pain patients (Janner et al., 2018).

During acute electric pain, I found less pain sensitivity on the right compared to the left arm, which could be caused by the right-handedness of all participants, for example, due to more muscle tissue in the right arm. Alternatively, the pain threshold assessment was performed only on the right arm and might have led to pain habituation effects.

According to the predator imminence model (Fanselow, 1994, 2018a), HR deceleration is described during the pre-encounter phase, i.e. anxiety, in anticipation of a distal threat. HR acceleration, which prepares the organism for fight or flight, could be observed in circa-strike phases when the threat was very close (Fanselow, 1994, 2018a). Interestingly, I found a similar HR pattern during the trials of electric pain. Here, participants might easily have learned that the painful electric stimulus was administered around 25 s after trial onset. The HR deceleration in anticipation of the electric stimulus could represent an defensive response associated with orienting reactions to the expected painful stimulus, whereas HR acceleration after the electric stimulus clearly describes the physiological preparation of the body for a fear response (Bradley et al., 2001) and the nociceptive response (Treister, Kliger, Zuckerman, Aryeh & Eisenberg, 2012).

Busch et al. (2013) and Kirchner et al. (2000) found subjective analgesic effects of tonic pain induced by tVNS and VNS, respectively. Busch et al. (2013) used a within design and applied tVNS or sham stimulation on two consecutive days while applying the quantitative sensory testing battery on both days. Such a design excludes any problems with group differences in participants' trait characteristics. However, they only used a non-stimulated control group, rather than a sham stimulation, which does not exclude contextual changes of a tingling sensation at the ear during pain perception. According to

the motivational priming hypothesis, a context indeed modulates subjective as well as electro-cortical pain processing (Kenntner-Mabiala & Pauli, 2005) and attentional processes (Wieser et al., 2012) during tonic and phasic pain. On the other hand, Kirchner et al. (2000) found analgesic effects only for tonic, but not for phasic pain stimulation. Attentional processes might explain these findings as attention towards the painful stimulus increases pain perception, whereas the attention to the periphery decreases pain sensation (Levine, Gordon, Smith & Fields, 1982; Wieser et al., 2012). In the current study, increasing pressure pain might have elevated the attention towards the tonic pain rather than the ear stimulation, whereas the expectation of a short electric stimulus could have attracted the participants' attention. To explore this experimentally, one could add an additional task, which attracts attention and test the performance.

Apart from the ear stimulation, I found reduced pain tolerance during pressure pain on the left compared to the right hand, which seems plausible because I only included right-handed mostly female participants, whose muscles are usually more pronounced on the dominant right hand resulting in higher resistance to the pressing stylus and therefore higher pain tolerance. In parallel, Brennum et al. (1989) reported higher pressure pain thresholds in the dominant compared to the non-dominant hand. Supportively, Göbel and Westphal (1987) found higher pain sensitivity in women on the non-dominant hand. However, participants seemed to habituate to the pressure pain as they reported less pain unpleasantness and showed decreased HR in the post1 phase of the experiment. The post2 phase is hard to compare with the previous phases as here the upper finger phalanxes were stimulated rather than the middle phalanxes in pre and post1. Though during pressure stimulation, the pain increased continuously, HR changed from deceleration to acceleration around 25 s after trial onset. Participants might have expected the highest pain in the same timing as the electric pain stimulus was previously

applied, namely 25 s after trial onset. A further explanation might be the onset of pain ratings 25 s after trial onset or the pressure pain was well bearable during the first 25 s and started to be painful in accordance with a physiological preparation for flight response around this time window. Exploratory comparisons of HR alterations during pressure pain stimulation before (0-15 s of the trial) and concurrently (15-30 s of the trial) with the respective ear stimulation revealed deceleration in the second interval for all groups. HR deceleration could indicate a functioning vagus nerve stimulation throughout our experiment. Speculatively, HR might not be the best option to depict the slow slope of increasing pain here (Treister et al., 2012). Alternatively, an orientation response in terms of HR deceleration to upcoming threat, i.e. increasing pressure pain, might be more likely due to the HR deceleration irrespective of tVNS or any control group (i.e. sham, control).

For the interpretation of the data, some study limitations should be considered. First, the phasic and tonic pain blocks were not randomized, which could have led to order effects of the painful stimulations as well as of the manipulation, i.e. tVNS, sham or control condition. The electric pain might have raised expectations on pain for the following pressure pain stimulation procedure. Therefore, the randomization of the blocks should be considered in the future. Second, the order of the painful stimuli to the left or right hand or arm, respectively, was also not counterbalanced but had a specific order. Third, since I hypothesized analgesic effects in the tVNS group with the same stimulation parameters that I have used in the previous anxiety conditioning study (Genheimer et al., 2017), only left ear stimulation was applied. However, Janner et al. (2018) reported analgesic effects using either left or right ear stimulation. Assuming no cardiac effects by tVNS on the right ear, future studies could implement tVNS on both ears. Fourth, in many participants the quality of physiological data was very low resulting in their exclusion

from HR analyses. Especially for physiological effects, a bigger sample is required in future studies.

To sum up, the current study was designed to provide evidence for the effectiveness of the tVNS stimulation employed in my previous study (Genheimer et al., 2017) by testing the analgesic effect found in earlier VNS and tVNS studies (Busch et al., 2013; Janner et al., 2018; Kirchner et al., 2000). However, with current stimulation parameters, I could neither replicate the analgesic effects of tVNS on pressure pain as reported by Busch et al. (2013), nor phasic pain reduction found by Janner et al. (2018). One conclusion could be that the stimulation with my described parameters has no effect at all, neither on subjective nor on physiological level of anxiety or pain. However, as many studies in animals, healthy humans and patients found memory-enhancing (Burger et al., 2017; Fanselow, 2013; Peña et al., 2013), anxiolytic (Peña et al., 2013) as well as analgesic effects (Busch et al., 2013; Kirchner et al., 2000; Mauskop, 2005) induced by VNS or tVNS, it is more likely that first, stimulation parameters have to be optimized for successful stimulation effects, and second, that a more reliable manipulation check for the effectiveness of tVNS has to be investigated and implemented.

5 Study 4: tVNS and Cue Conditioning

5.1 Introduction

So far, tVNS has had the expected effects neither on extinction nor on retrieval of anxiety or pain perception. Those inconsistent conditioning findings could result from different designs. Study 2 contained a context conditioning procedure, whereas Burger et al. (2016) used a cue conditioning design. However, fear and anxiety acquisition and extinction are more complex procedures than the pure model of cue or context conditioning considers. Therefore, I aimed to investigate an even more realistic, but well-controlled cue in context conditioning model in VR with altered tVNS application for optimization of extinction learning outcome and attenuated return of fear.

Three main factors could have been the reason for our findings in the previous tVNS studies: The chosen stimulation parameters, the implementation of tVNS into the experimental design, and the preferred paradigm to model anxiety. First, the most effective stimulation parameters of tVNS, particularly in humans, are still unclear. In both tVNS studies we considered the temporal latency of the effects in the human brain described by Frangos et al. (2015). Indeed, they found an increase in NTS activity 7 min after constant tVNS stimulation (Frangos et al., 2015). However, in comparison to epilepsy patients wearing an implanted VNS for a couple of months or years and applying stimulation regularly, our stimulation time of several minutes is still very short (see meta-analysis by Englot, Chang & Auguste, 2011). Regarding extinction training, the animal studies by Peña et al. (2014; 2013) both started the invasive stimulation 150 ms before stimulus onset and ended with stimulus offset. George et al. (2008), who investigated the effects of an implanted VNS in anxiety patients, used a 10 weeks interval of stimulation

during their study and found remarkable treatment effects in some patients. In contrast, investigations using tVNS only used intervals between 0 and 20 min of either constant or interval stimulation before extinction and during extinction, which makes a total stimulation time of 60 to 120 min (Burger et al., 2017; Burger et al., 2016; Genheimer et al., 2017). Interestingly, Fang et al. (2017) prolonged tVNS in patients suffering from major depressive disorder (MDD). After instruction, patients stimulated themselves for four weeks, twice a day, at least five days a week. Improvements in Hamilton Depression Rating Scale (HAM-D; Hamilton, 1986) scores in verum compared to sham stimulated patients speak for an effective stimulation procedure. Here, prolonged stimulation time could facilitate not only neurotransmitter release in the brain over an extended period but could also result in neurobiological adaptions in receptor development of the corresponding cells and neural plasticity. To this end, future studies are required to consider prolonged stimulation, even though this would be an enormous effort. In the current study, I aimed to extend the stimulation time to two times 30 min prior and in between extinction as well as during extinction. Furthermore, I applied a context-dependent cue conditioning rather than a pure context conditioning paradigm since successful tVNS has been revealed in cue conditioning (Burger et al., 2016).

The stimulation context could be the second reason for our previous findings on tVNS. In the first tVNS study (Study 2), I only applied the stimulation on the second day during extinction, which created a distinct change in the context compared to acquisition and re-extinction without stimulation (Genheimer et al., 2017). As many studies in animals and humans have revealed that extinction is highly context-dependent (e.g. Bouton et al., 2006; Kalisch et al., 2006; Vansteenwegen, 2005) a slight change in the study design is suggested for future investigations to keep the context of acquisition and extinction as similar as possible. Furthermore, in an ABA design, it was shown that anxiety returns

more likely when extinction was performed in a different context as acquisition and return of anxiety context (Vansteenwegen, 2005). Notably, Pena et al. (2014; 2013), as well as Burger et al. (2016), used stimulation only paired with extinction training. Despite controlling for such context changes evoked by the stimulation by adding a third group without stimulation (Genheimer et al., 2017), the more convenient, economic, and adequate way would be to stick with two groups that were also stimulated during acquisition and re-extinction or test of relapse, respectively. Burger et al. (2017) found an elegant solution by sham stimulating all participants during acquisition and re-extinction at the earlobe. Only during extinction, the sham group got sham stimulation whereas the experimental group got verum stimulation. In this way, the context changed minimally for the experimental group, due to the different stimulation sites, but the tingling sensation and therefore the overall context stayed the same.

The third main difference between our and other tVNS findings is the paradigm. Some existing VNS or tVNS studies on extinction and retrieval in animals and humans investigated fear and some investigated anxiety (Burger et al., 2017; Burger et al., 2016; Genheimer et al., 2017; Peña et al., 2014; Peña et al., 2013). However, the examination of the different effects of tVNS on cue and context extinction is unclear and could be the reason for the mixed results found in the literature. Substantially, Burger et al. (2016) used a cue conditioning paradigm and found accelerated declarative memory shown by lower contingency ratings for CS+ in the tVNS compared to the sham group. Though in their study Burger et al. (2017) refer to fear conditioning, they present the geometric shapes, which served as CSs, for 30 s and US administration during CS+ was unpredictable indicating contextual anxiety conditioning, rather than fear conditioning. In parallel to their previous findings (Burger et al., 2016), tVNS improved the declarative memory, but not psychophysiological responses (Burger et al., 2017). Considering the two-factor

model of emotional memory by Phelps (2004), the emotional load of an event is mainly processed in the amygdala, whereas the declarative memory of an event is formed by the hippocampus. In parallel, the hippocampus is both highly involved in context conditioning and extinction (see Milad & Quirk, 2012) as well as indirectly affected by tVNS (Çalışkan & Albrecht, 2013). A combinational approach of cue and context conditioning and tVNS could shed light on the interaction between cue and context conditioning and the inconsistent effects of tVNS in human extinction.

Mühlberger et al. (2014) used a combined cue and context conditioning paradigm to investigate the single nucleotide polymorphism in the brain-derived neurotrophic factor (*BDNF*) gene. They used two contexts in VR serving as CTX+ and CTX- as well as two colored lights, which served as CS+ and CS-. Please note, during acquisition, CS+ was only followed by an US, when presented in CTX+. Accordingly, startle response was potentiated to CS+ in CTX+ as compared to CS- as well as CS+ in CTX-. Interestingly in a test phase, carriers of one *BDNF* Met variant, which has been related to high risk for anxiety disorders, showed potentiated startle responses to CS+ presented in a novel context suggesting generalization of conditioned fear. Changes in the dendritic morphology in the hippocampus might result in impaired context-specific learning. As the fear memory is encoded context-dependent, the no-risk group (Val/Val carriers) associated the CS+ with threat only in the anxiety context. Likewise, a recent study by Andreatta, Genheimer, Neueder, Wieser, and Pauli (submitted-a) reported differentiated fear responses in ratings, skin conductance and startle response for CS+ compared to CS-, but only when they were presented in the anxiety rather than the safety context, which indicated context-specific fear learning. However, when CS+ and CS- were presented in a novel context, which is the equal mix of CTX+ and CTX-, startle responses were increased for CS+ versus CS- again indicating generalization of conditioned fear.

Considerably, high compared to low anxious individuals (measured by ASI; Alpers & Pauli, 2001) tend to generalize their subjective fear to stimuli with similar physical properties (Baumann et al., 2017). Baumann et al. (2017) found lower valence and higher US expectancy ratings for faces that were similar to the face representing CS+ in high compared to low anxious individuals. The clinical relevance of generalization research showed investigations of anxiety patients. Lissek et al. (2009) and Jovanovic et al. (2012) described the inability of safety learning in PD and GAD patients. Consequently, patients tend to generalize their fear to stimuli that share some properties with the originally threatening stimulus but have never been associated with any kind of threat (Dunsmoor & Paz, 2015; Dymond et al., 2015; Lissek et al., 2014). Additionally, patients suffering from social anxiety disorder (SAD) showed fear generalization on heart rate level compared to healthy controls (Ahrens et al., 2016). All these patients could benefit from research on the improvement of extinction learning in several ways. First, exposure therapy could be accelerated and improved. Second, the return of fear or anxiety could be attenuated. Third, overgeneralization could be hampered by improved safety learning. A stronger extinction memory might lead to a clear differentiation between a formerly threat associated stimulus and a similar stimulus that has not been paired with a threat.

While a lot of research on fear generalization exists (Baumann et al., 2017; Dunsmoor & Paz, 2015; Dymond et al., 2015; Lissek et al., 2008), investigations of the generalization of anxiety are sparse. Andreatta et al. (2015b) used the same offices in VR that were used in Genheimer et al. (2017). Additionally, they created a third context (generalization context, GCTX), which was again the equal mix of both other contexts. After acquisition with CTX+ and CTX-, healthy participants reported more arousal and anxiety in GCTX than in CTX- indicating anxiety generalization, but they showed potentiated startle responses only in CTX+ compared to GCTX and CTX-. Though GCTX only consisted of 50% CTX+, on

an explicit level, participants generalized conditioned anxiety. In patients, generalization is expected also on an implicit level. Anxiety patients could benefit from strong extinction to more specifically learn CS-noUS association, i.e. extinction, and therefore to be less susceptible to the generalization of their anxiety triggering stimuli.

Altogether, the goal of the current study was to investigate the prolonged and well-controlled tVNS effects on extinction and generalization in a context-dependent cue conditioning paradigm. Therefore, I combined knowledge gained from previous tVNS studies by Burger et al. (2017), Fang et al. (2017), and Genheimer et al. (2017), with the knowledge about cue in context conditioning paradigm by Mühlberger et al. (2014) and Andreatta et al. (submitted-a; 2015b). Regarding the ear stimulation, I presumed HR deceleration for tVNS compared to sham stimulated participants during extinction learning. Subsequently, I expected successful context-dependent cue conditioning depicted in potentiated startle responses and increased anxiety ratings in CTX+ when CS+ compared to CS- was present and compared to CTX-. Additionally, I hypothesized accelerated extinction learning, reduced reinstatement and lower fear generalization in the tVNS compared to the sham stimulated group, which should be indicated on the physiological level, i.e. startle response, as well as on subjective level, i.e. valence, arousal, fear, and contingency ratings. Moreover, the design enables the additional test of pure context conditioning similar to Study 2. Therefore, I hypothesized successful context conditioning depicted in potentiated startle responses in CTX+ compared to CTX-, faster extinction, lower reinstatement and less anxiety generalization for the tVNS compared to the sham stimulated group. With this, I aimed to gain information on proper stimulation parameters, to increase our knowledge on tVNS effects in healthy participants, and to extend previous findings to generalization research.

5.2 Material and methods

Kathrin Delius, Marlena Milzer and Elena Rüsch wrote their Bachelor Theses within the scope of this study under my supervision.

5.2.1 Participants

In total, 64 participants took part in the experiment. Twelve participants had to be excluded: four participants quit the experiment early because of cybersickness or dizziness, and physiological data of eight additional participants were insufficiently recorded and therefore excluded from analyses. To this end, datasets of 52 participants, 27 in the tVNS group and 25 in the sham group, were analyzed. Groups neither differed in gender nor the number of aware participants, nor in subjective and objective ear stimulation or US intensities (Table 10).

Table 10: Sample characteristics of Study 4 separated into groups.

	tVNS	**sham**	**statistics**
N (females)	27 (14)	25 (13)	$\chi^2(1) = .991$
Age (*SD*)	24.0 (4.5)	23.9 (4.7)	$t(50) = 0.10$, $p = .925$
Aware (N)	21	19	$\chi^2(1) = .879$
Stimulation intensity [mA] (*SD*) pre Acq Day1	1.3 (1.0)	1.0 (0.7)	$t(50) = 1.26$, $p = .214$
Stimulation rating (*SD*) pre Acq Day1	6.5 (0.5)	6.3 (0.5)	$t(50) = 1.50$, $p = .139$
Stimulation intensity [mA] (*SD*) pre Ext Day1	1.1 (0.9)	1.2 (0.7)	$t(50) = 0.52$, $p = .605$
Stimulation rating (*SD*) pre Ext Day1	6.5 (0.6)	6.2 (0.5)	$t(50) = 1.88$, $p = .066$
Stimulation intensity [mA] (*SD*) pre Gen Day2	1.2 (0.8)	1.1 (0.5)	$t(50) = 0.79$, $p = .435$
Stimulation rating (*SD*) pre Gen Day2	6.4 (0.5)	6.5 (0.5)	$t(50) = 0.80$, $p = .426$
US intensity [mA] (*SD*)	1.6 (0.8)	1.7 (1.0)	$t(50) = 0.40$, $p = .690$
US rating	5.7 (0.7)	5.6 (0.7)	$t(50) = 0.95$, $p = .345$

5.2.2 Stimulus Material

Virtual reality. We applied the same technology of VR as used in Study 2, i.e. a VR environment of two comparable offices (CTX+, CTX-) connected by a corridor (ITI), which was created with Source Engine (Valve Corporation, Bellevue, USA). For the generalization test, an additional context (GCTX) was used, made of 50% of one and 50% of the other office, and was also connected to the other rooms via the corridor. In addition to the offices, I used two colored lights, yellow and blue, as cues (CS+, CS-), which enlightened the whole context for 8 s when they were switched on (Andreatta et al., submitted-a; Mühlberger et al., 2014). In addition to the Powerwall, we used head tracking and 3D presentation of the contexts to increase the presence feeling of the participants. The experiment was controlled by Cybersession software (CS-Research 5.6, VTplus GmbH, Würzburg, Germany; see www.cybersession.info for detailed information).

Unconditioned Stimulus. Mildly painful electric stimuli were used as the unconditioned stimulus. The stimuli of 200 ms were individually determined and delivered with a bar electrode on the right inner forearm (Digitimer DS7A, Digitimer LTD., Welwyn Garden City, UK). The exact pain threshold procedure has already been explained above.

Vagus nerve stimulation. For the application of transcutaneous vagus nerve stimulation NEMOS stimulator (cerbomed GmbH, Erlangen, Germany) was used with the same parameters as in the studies described above. Verum stimulation was applied at the cymba concha, whereas sham stimulation was applied at the helix of the outer ear. However, the determination of the participant's individual stimulation intensity was performed differently than described above. The experimenter applied the tVNS device according to tVNS or sham condition. Then participants saw the 11-point Likert scale for tVNS ranging from 0 (no sensation) to 3 (slight tingling) to 6 (strong tingling) to 10 (pain). The experimenter switched on the tVNS, covered the stimulation intensity number on the

stimulator and handed the device to the participant who was asked to increase and decrease the intensity on the arrow buttons until they rated a 7 on the stimulation scale. The experimenter noted the intensity, restarted the stimulation with 0.1 mA and the participants were again asked to adjust the intensity until they rate a 7 on the scale. Subsequently, the experimenter noted the intensity, calculated the mean of both determined stimulation intensities rated with 7 and applied stimulation with this intensity. Participants rated their tingling sensation again on the stimulation scale. When the rating was 6 or 7, the intensity was used for the subsequent phase of the experiment.

5.2.3 Measures

Questionnaires. Before participants were invited to take part in the study, an interview by phone was conducted to inquire participants' health conditions according to previously described parameters, to exclude prior experience with vagus nerve stimulation and the virtual reality stimuli, and to make an appointment. In the course of the experiment, a demographic questionnaire, the STAI (German version by Laux et al., 1981), PANAS (German version by Krohne et al., 1996), ASI (German version by Alpers & Pauli, 2001), IPQ (Schubert et al., 2001), and a follow-up questionnaire about the vagus nerve stimulation were used (see Studies 2 and 3). Additionally, the Intolerance of Uncertainty (IU; Dietmaier, Ille, Schäfer, Leutgeb & Schienle, 2008; Gerlach, Andor & Patzelt, 2008) and the Minnesota Multiphasic Personality Inventory (MMPI; Hank & Schwenkmezger, 2003) were assessed and a memory test was conducted.

Ratings. Similar to Study 2, ratings of valence, arousal, anxiety, and contingency were assessed on a 100-point rating scale presented on the Powerwall and participants had to verbally report their rating. Besides the ratings of the contexts alone, additional ratings of

the contexts in the colored lights were assessed. For all ratings, a picture of the referring context and light was depicted.

Startle. As reported in Study 2, startle probes contained a 103 dB loud white noise for 50 ms. The resulting EMG was derived from two electrodes fixed on the *M. orbicularis oculi* according to Blumenthal et al. (2005). Reference and ground electrodes were attached at the forehead and left mastoid, respectively. Vision Recorder software (version 1.21, Brain Products Inc., Munich, Germany) continuously recorded the physiological data with a sampling rate of 1000 Hz and an online Notch filter of 50 Hz.

HR. As a manipulation check for successful transcutaneous vagus nerve stimulation, we analyzed participants' heart rate during extinction learning. HR was recorded as described above. We cleaned participants' skin on the right clavicle and the left lower costal arch with alcohol and attached adhesive Ag/AgCl foam electrodes of 55 mm diameter by Swaromed. Data were recorded with Vision Recorder software (version 1.21, Brain Products Inc., Munich, Germany) with a sampling rate of 1000 Hz and a Notch filter of 50 Hz.

5.2.4 Procedure and Design

Before participants were invited to take part in the experiment, they were interviewed by phone and screened regarding inclusion criteria of age (18-35 years), no excessive alcohol, smoking or other drug consumption, neurological and psychological health, no pregnancy, neither participation in a similar VR experiment nor pre-experience with vagus nerve stimulation. When a participant met all criteria, he or she was invited to the laboratory on two consecutive days. Participants first read the study instructions, signed the written informed consent and filled-in a demographic questionnaire, STAI state (Laux et al., 1981), and PANAS (Krohne et al., 1996). Afterward, electrodes for startle were

applied, electrodes for the mildly painful electrical stimulus were fixed at participants' right inner forearm and the transcutaneous vagus nerve stimulator was attached for all participants to the helix of the ear for sham stimulation during acquisition. Subsequently, the procedure for the determination of the pain threshold and the tVNS intensity was performed as described above. From there on, sham stimulation was conducted in 30 s on/off cycles for the entire habituation and acquisition phase. During habituation, participants could freely explore the two contexts for two minutes each by moving around with a joystick (for an overview, see Figure 16). While participants were in the offices, once a yellow and once a blue light was turned on for 8 s each. After this phase, participants rated valence, arousal and anxiety regarding both offices and regarding the offices appearing in yellow and blue light. Before the acquisition, 7 startle probes were randomly presented to avoid initial startle reactivity. Subsequently, an acquisition phase was conducted consisting of four trials in which participants were passively moved through one context and four trials in the other context in a pseudo-randomized order, i.e. one context was at most presented twice in a row. Please note that acquisition was split into two phases for startle analysis, i.e. early (A1) and late acquisition (A2). One trial started and ended in the corridor in front of an office, the time a participant spent in an office was 3 min. At that, three times each colored light was switched on for 8 s in randomized order. In case the participant was in the anxiety context (CTX+), the offset of one colored light (CS+) was paired with a mildly painful electrical stimulus (US), but not the offset of the other light (CS-). When the participant was guided through the safety context (CTX-), both lights (CS+ and CS-) were presented as in CTX+ but without US delivery. In total, 12 CS+ were presented in CTX+, 12 US were administered during acquisition. Six startle probes were presented in each of the 6 conditions, i.e. CTX+, CTX+CS+, CTX+CS-, CTX-, CTX-CS+, CTX-CS-. In the corridor (ITI), 4 startle probes were

presented. After the acquisition, participants rated valence, arousal, fear/anxiety, and contingency for all conditions.

Afterward, the vagus nerve stimulator was removed and reinserted depending on the participant's affiliation to tVNS or sham group. Again, the stimulation threshold was determined for all participants according to the same procedure described above. Then, a 30 min stimulation phase consisting of 30 s on/off cycles was applied. In the meanwhile, participants filled in questionnaires of STAI trait (Laux et al., 1981), ASI (Alpers & Pauli, 2001), IU (Dietmaier et al., 2008; Gerlach et al., 2008), MMPI (Hank & Schwenkmezger, 2003). Subsequently, the first extinction phase (E1) started in the same way as the first half of the acquisition phase but without US delivery. Then, participants rated all conditions according to valence, arousal, fear/anxiety, and contingency. Afterward, a second 30 min stimulation interval with questionnaire completion was conducted, followed by Extinction 2 (E2) and ratings. At the end of the first day, STAI state (Laux et al., 1981) and PANAS (Krohne et al., 1996) were filled in.

Day 2 started with the completion of STAI state (Laux et al., 1981) and PANAS (Krohne et al., 1996), and the attachment of all electrodes as on Day 1. For all participants, sham vagus nerve stimulation was applied during the experiment with a newly determined stimulation intensity. At the beginning of the second day of the experiment, three US with an inter-stimulus-interval of 6-8 s were delivered for reinstatement while participants saw a black screen on the Powerwall. Ratings of all conditions followed. During the test phase, participants were twice guided through CTX+, CTX-, and a third generalization context (GCTX). The lights were switched on and off in all three contexts similar to the procedure on Day 1. Therefore, five startle probes were presented for each condition (CTX+, CTX+CS+, CTX+CS-, CTX-, CTX-CS+, CTX-CS-, GCTX, GCTX-CS+, GCTX-CS-). The ITI contained four startle probes. At the end of Day 2, STAI state (Laux et al., 1981), PANAS

(Krohne et al., 1996), IPQ (Schubert et al., 2001), and a follow-up questionnaire on participants' subjective experience with the vagus nerve stimulation were completed.

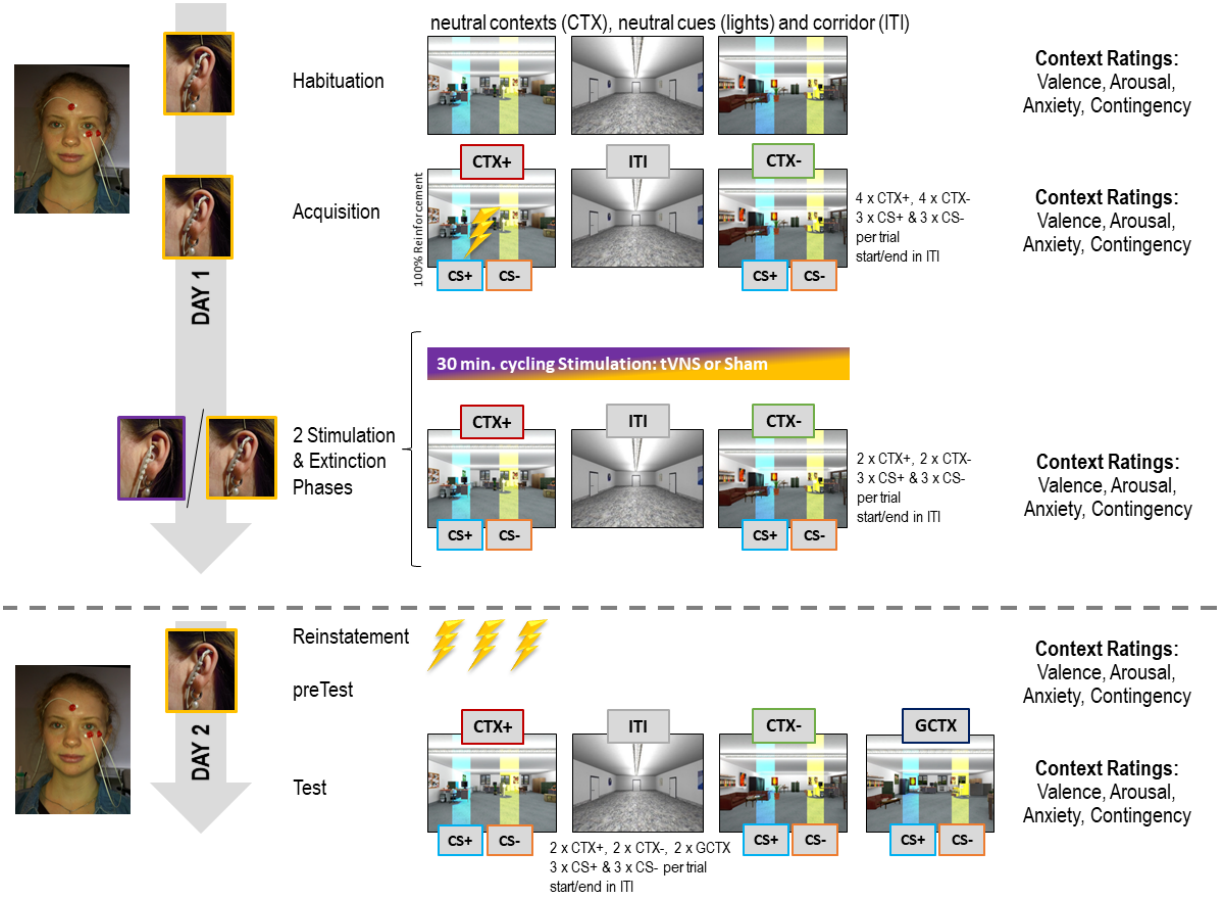

Figure 16: Experimental design of Study 4.

All participants received sham stimulation and underwent a habituation and acquisition phase, in which one of the lights (CS+) was paired with an electric stimulus (US) only in one of the virtual contexts (CTX+). The other light (CS-) and both lights (CS+ and CS-) in the second context (CTX-) were not associated with the US. Afterwards, a 30 min interval stimulation of either tVNS or sham was applied and accompanied the extinction phase 1 without US delivery. This was repeated twice. On the second day, all participants received sham stimulation. Three USs were applied for reinstatement and a test phase with an additional generalization context (GCTX) was performed. Startle responses and subjective ratings of valence, arousal, anxiety/fear and contingency were assessed.

5.2.5 Data Recordings and Data Reduction

Startle. Data processing was performed with Vision Analyzer 2.1 software (Brain Products Inc., Munich, Germany) as already described in Study 2. Accounting for individual differences in startle scores, *z*-transformation was conducted with raw data

within subjects and subsequently transferred to T-scores. Startle data were interpolated for participants who had at least one remaining startle probe per condition, phase, and group. The interpolation was performed by calculating the mean values of the T-scores of each single startle response per condition separately for tVNS and sham group.

HR. Data processing was performed with Vision Analyzer 2.1 software (Brain Products Inc., Munich, Germany) as described in Study 2. Data of the extinction phases were cut into segments of 180 s, starting and ending with the individual context trials. The mean HR per condition, i.e. CTX+ and CTX- and trial, i.e. Extinction Trial 1-4, were exported for each participant for statistical analyses in SPSS.

5.2.6 Statistical Analyses

All statistical analyses were performed with SPSS (Version 24.0.0.1, SPSS Inc.). The state questionnaires were analyzed with repeated measures ANOVAs including the within-subject factor time (beginning of Day 1, end of Day 1, beginning of Day 2, end of Day 2) and the between-subjects factor group (tVNS, sham).

Manipulation check of tVNS by HR: To control for successful physiological effects of tVNS, we calculated a mixed 2 x 4 x 2 ANOVA with the within-subject factors context (CTX+, CTX-) and trial (Ext1, Ext2, Ext3, Ext4) and with the between-subjects factor group (tVNS, sham). Significant interactions were resolved with post-hoc t-tests.

According to my a priori hypotheses on context-dependent cue conditioning, simple contrasts were calculated, and Bonferroni corrected according to the number of tests. During acquisition, startle responses to CS+ and CS- were compared in CTX+ and CTX- separately for A1 and A2. Ratings of CS+ and CS- were compared for CTX+ and CTX- after the acquisition phase. For extinction, startle responses and ratings were compared between CS+ and CS- for each context, i.e. CTX+ and CTX-, and each phase, i.e. E1 and E2,

separately for each group. The reinstatement test for startle responses was calculated by contrasts during the test phase for the first two startle responses on CS+ and CS- in CTX+ and CTX- for each group, respectively. Reinstatement of all ratings was analyzed by comparing CS+ and CS- after reinstatement separately for each context and group. For generalization, startle responses and ratings on CS+ and CS- were compared in CTX+, CTX- and GCTX separately for each group.

Similarly, according to my context conditioning hypotheses, simple contrasts were calculated for startle responses in CTX+, CTX- and ITI during A1 and A2, during E1 and E2, during end of extinction (endE) and start of the test (startT) for reinstatement, and for CTX+, CTX- and GCTX for generalization. For the ratings, simple contrasts were calculated between CTX+ and CTX- for Hab, Acq, E1, and E2 as well as after reinstatement (Reinst). Generalization was tested by contrasts of CTX+, CTX- and GCTX.

5.3 Results

5.3.1 Questionnaires

The analysis of participants‘ positive affect revealed a main effect of time ($F(3,150) = 9.36$, GG-$\varepsilon = .800$, $p < .001$, $\eta_p^2 = .158$), which indicated more positive affect at the beginning than at the end of both Day 1 ($t(51) = 4.28$, $p < .001$) and Day 2 ($t(51) = 2.66$, $p = .010$). The positive affect at the beginning of Day 2 was higher than at the end of Day 1 ($t(51) = 3.42$, $p = .001$). Neither the main effect group nor the Time x Group interaction effect reached a significant level (all *p*s > .593). Moreover, the investigation of the negative affect revealed neither a significant main effect of time nor group nor an interaction Time x Group (all *p*s > .716), which indicated stable negative affect of the participants throughout the entire experiment. The analysis of the STAI state questionnaire

demonstrated neither differences in time nor group nor in the interaction of Time x Group (all *p*s > .260).

The analyses of the trait questionnaires revealed neither group differences (all *p*s > .324) nor remarkably increased mean scores (see Table 11).

Table 11: Trait questionnaires of Study 4 compared between groups.

	tVNS	**sham**	**statistics**
STAI trait (*SD*)	38.59 (7.70)	39.20 (11.49)	$t(50) = 0.23$, $p = .823$
ASI (*SD*)	18.41 (9.81)	17.68 (10.36)	$t(50) = 0.26$, $p = .796$
IU (*SD*)	27.59 (6.89)	25.76 (8.93)	$t(50) = 0.83$, $p = .409$
IPQ General Presence (*SD*)	3.04 (1.37)	3.08 (1.86)	$t(50) = 0.10$, $p = .920$
IPQ Spatial Presence (*SD*)	3.16 (1.07)	3.34 (1.39)	$t(50) = 0.52$, $p = .603$
IPQ Involvement (*SD*)	2.45 (1.42)	2.50 (1.58)	$t(50) = 0.11$, $p = .912$
IPQ experienced Realism (*SD*)	1.86 (1.10)	2.16 (1.06)	$t(50) = 0.99$, $p = .325$

5.3.2 Stimulation conditions

Similar to Studies 2 and 3, in the tVNS questionnaire participants rated their own experience by the ear stimulator and whether they felt any side effects of the stimulation. The groups did differ in neither of the variables (all *p*s > .514, see Table 12).

Table 12: Rating of stimulation in tVNS and sham group in Study 4.

	tVNS	Sham	statistics
Conviction (*SD*)	5.78 (2.67)	5.84 (2.29)	$t(50) = 0.90$, $p = .929$
Operability (*SD*)	6.70 (2.60)	6.36 (3.75)	$t(50) = 0.38$, $p = .705$
Valence (*SD*)	4.81 (1.62)	5.16 (2.15)	$t(50) = 0.66$, $p = .515$
Clinical use (*SD*)	7.04 (2.52)	6.60 (2.48)	$t(50) = 0.63$, $p = .532$
Side effects (*N*)	5	3	$\chi^2(1) = .515$

The side effects, which participants reported, included calming, increased attention, slight dizziness, tingling, and pricking sensation still some hours after the experiment, tension, skin irritation, fatigue, and slight nausea.

5.3.3 Manipulation check of tVNS by HR

The ANOVA[9] comparing heart rate between both groups revealed a main effect of extinction trial ($F(3,138) = 7.70$, $GG\text{-}\varepsilon = .704$, $p = .001$, $\eta_p^2 = .143$) and an interaction of Extinction Trial x Context ($F(3,138) = 7.26$, $GG\text{-}\varepsilon = .704$, $p < .001$, $\eta_p^2 = .136$). Post-hoc *t*-tests resolving the interaction indicated lower HR in CTX+ compared to CTX- in the first extinction trial ($t(47) = 2.44$, $p = .018$), similar HR during the second extinction trial ($t(47) = 0.44$, $p = .608$), and lower HR in CTX- compared to CTX+ in the third and fourth extinction trial ($t(47) = 3.60$, $p = .001$; $t(47) = 2.53$, $p = .015$, respectively). All other comparisons did

[9] Please note that four participants had to be excluded from HR analysis due to deficient recordings of the electrocardiogram (1 from tVNS group, 3 from sham group). Therefore, HR analyses were performed with the resulting 26 participants in the tVNS group and 22 participants in the sham group.

not reach significance level (all *p*s > .050). Interestingly, the interaction of Context x Group just failed to reach the significant level ($F(3,138) = 4.01$, $p = .051$, $\eta_p^2 = .080$). According to my a priori hypothesis of decelerated HR due to tVNS compared to sham stimulation, I further compared HR in CTX+ with CTX- for tVNS ($t(25) = 0.22$, $p = .825$) and sham stimulated participants ($t(21) = 2.23$, $p = .037$), separately, and found decelerated HR in CTX- in the sham, but not in the tVNS group. This is in contrast to my hypothesis and again raises the question whether the here used parameters are appropriate to successfully stimulate the auricular branch of the vagus nerve in humans.

5.3.4 Context-dependent cue conditioning

Habituation.

Ratings. Ratings of the colored lights, which were later used as CS+ and CS-, did not return any differences (all *p*s > .154; Figure 17B-D) regarding valence, arousal, and fear. This indicates no baseline preferences for one CS over the other. Under that premise, the stimuli can be used as CS for conditioning.

Acquisition.

Startle. In A1, startle responses were similar for CS+ and CS- in both contexts (all *p*s > .774). Though calculated contrasts for A2 did not survive Bonferroni corrections (4 comparisons, $p < .013$), startle responses during CS+ were descriptively potentiated compared to CS- in CTX+ ($F(1,51) = 4.12$, $p = .048$, $\eta_p^2 = .075$) as well as in CTX- ($F(1,51) = 5.06$, $p = .029$, $\eta_p^2 = .090$; see Figure 17A). Therefore, I could not assume cue conditioning, but demonstrated similarity to classical findings on simpler cue conditioning.

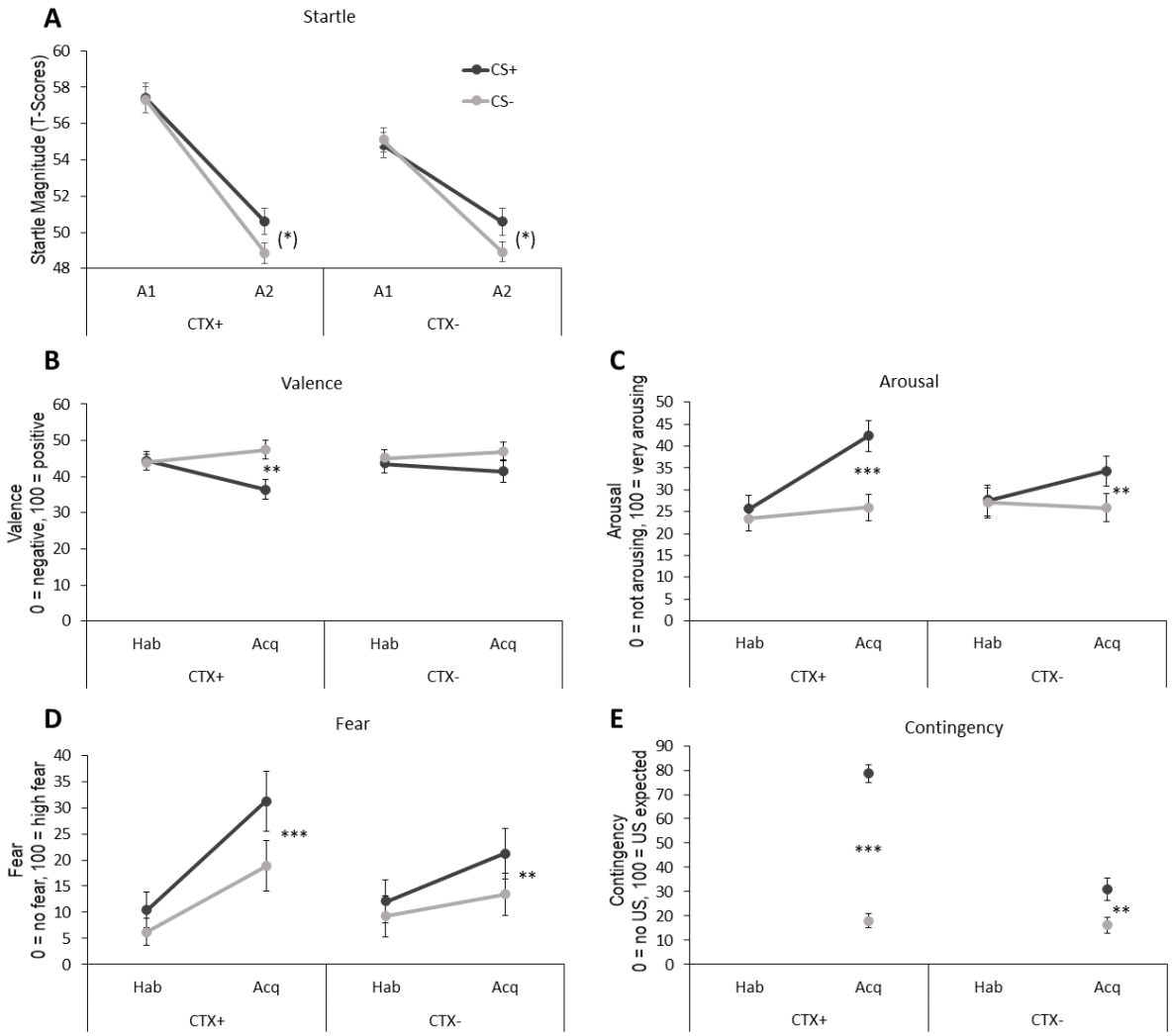

Figure 17: Habituation and acquisition of cue conditioning in Study 4.

Depicted are startle responses and standard errors (SE) for CS+ (black lines) and CS- (dark gray lines) during both acquisition phases (A1 and A2) for anxiety context (CTX+, left) and safety context (CTX-, right) separately (A). Valence (B), arousal (C) and fear ratings (D) for CS+ and CS- including standard errors (SE) are shown separately for CTX+ and CTX- after the habituation phase (Hab) and after the acquisition phase (Acq). (E) shows the respective contingency ratings after acquisition (Acq) for CTX+ and CTX-. *: $p < .05$; **: $p < .01$; ***: $p < .001$. Stars in brackets do not survive Bonferroni correction.

Ratings. CS+ was rated as more negative compared to CS- in CTX+ ($F(1,51) = 7.59$, $p = .008$, $\eta_p^2 = .130$), but equally pleasant in CTX- ($F(1,51) = 2.02$, $p = .161$, $\eta_p^2 = .038$). Interestingly, independent of context, CS+ compared to CS- was rated as higher arousing (CTX+: $F(1,51) = 22.85$, $p < .001$, $\eta_p^2 = .309$; CTX-: $F(1,51) = 8.38$, $p = .006$, $\eta_p^2 = .141$), more fear eliciting (CTX+: $F(1,51) = 14.07$, $p < .001$, $\eta_p^2 = .216$; CTX-: $F(1,51) = 8.38$, $p = .006$, $\eta_p^2 = .141$), and with increased US contingency (CTX+: $F(1,51) = 114.51$, $p < .001$, $\eta_p^2 = .692$;

CTX-: $F(1,51) = 11.43$, $p = .001$, $\eta_p^2 = .183$). Due to two tests per dependent variable, Bonferroni correction of $p < .025$ was applied. The results are illustrated in Figure 17B-E.

Extinction.

Startle. The tVNS group still showed a tendency of potentiated startle responses for CS+ compared to CS- in CTX+ during extinction (E1: $F(1,26) = 4.73$, $p = .039$, $\eta_p^2 = .154$; E2: $F(1,26) = 5.41$, $p = .028$, $\eta_p^2 = .172$; see Figure 18A). In CTX-, CS+ was significantly increased compared to CS- in E1 ($F(1,26) = 10.37$, $p = .003$, $\eta_p^2 = .285$), but not in E2 ($F(1,26) = 0.30$, $p = .590$, $\eta_p^2 = .011$). The sham group showed no significant startle differences between CS+ and CS- in CTX+ and CTX- during both extinction phases (all *p*s > .583). The results indicated slower extinction learning in the tVNS compared to the sham group on physiological level. After Bonferroni-corrections for 8 contrasts, $p < .006$ became statistically significant.

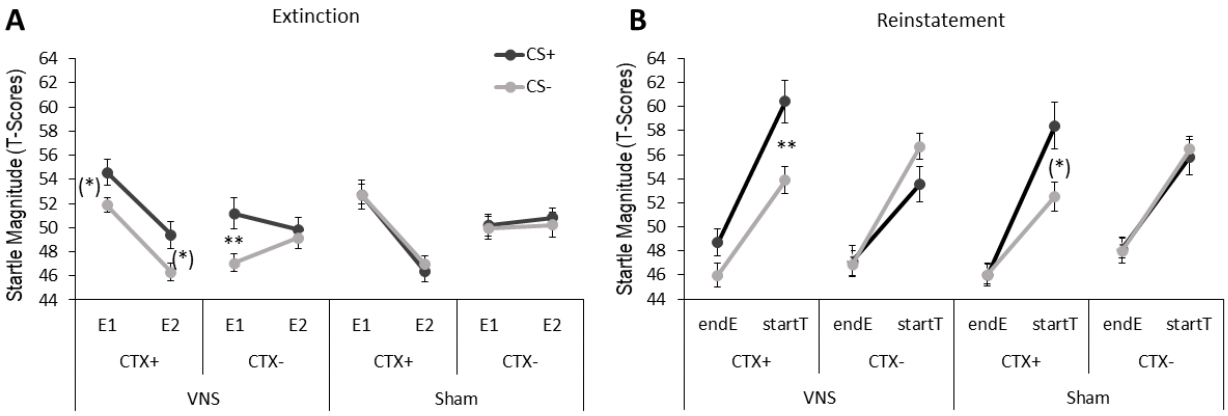

Figure 18: Startle responses to cues during extinction and reinstatement in Study 4.

(A) depicts startle magnitudes and standard errors (SE) in response to CS+ (black line) and CS- (dark gray line) separated for the anxiety context (CTX+, left) and safety context (CTX-, right) for the tVNS group (left) and the sham group (right) during extinction phases 1 and 2 (E1, E2). Respectively, reinstatement is shown in (B) by the illustration of the last 2 startle responses during extinction compared to the first two startle responses during test separated for context and group. *: $p < .05$; **: $p < .01$; ***: $p < .001$. Stars in brackets do not survive Bonferroni correction.

Ratings. In contrast to physiology, during extinction valence ratings of the tVNS group were similar for CS+ and CS- in both contexts (all *p*s > .122). In the sham group, more negative valence was assessed for CS+ compared to CS- in CTX+ after E1 ($F(1,24) = 12.63$, $p = .002$, $\eta_p^2 = .345$), but this effect disappeared after E2 ($F(1,24) = 0.35$, $p = .560$, $\eta_p^2 = .014$). No differences between CSs were found in CTX- (all *p*s > .077). The arousal ratings returned a similar pattern, which is shown in Figure 19A (Bonferroni-corrected $p < .006$).

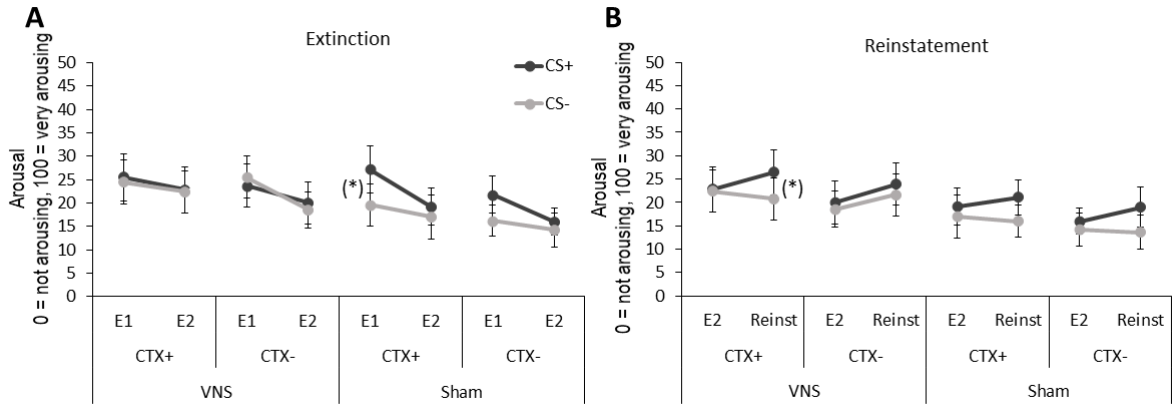

Figure 19: Arousal ratings of cues after extinction and reinstatement in Study 4.

Arousal ratings and standard errors (SE) for CS+ (black lines) and CS- (dark gray lines) are depicted separately for the anxiety context (CTX+, left) and the safety context (CTX-, right) for the tVNS group (left) and the sham group (right). (A) illustrates the ratings after the extinction phases 1 and 2 (E1, E2). (B) illustrates reinstatement effects by depicting arousal ratings after extinction (E2) and after reinstatement (Reinst). *: $p < .05$. Stars in brackets do not survive Bonferroni correction.

No differences between CSs were found in the tVNS group, neither in CTX+ nor in CTX- (all *p*s >.570). The sham group showed increased arousal ratings for CS+ compared to CS- in CTX+ after E1, but statistics did not survive Bonferroni correction ($F(1,24) = 4.38$, $p = .047$, $\eta_p^2 = .154$). Neither contrasts of CS+ and CS- in CTX- after E1 nor between CSs after E2 were significantly different (all *p*s > .062). Fear ratings of CS+ and CS- differed neither in the tVNS nor in the sham group independent of contexts (all *p*s > .137). Differences in

contingency ratings between CS+ and CS- were not significant after Bonferroni corrections, neither in tVNS nor in the sham group (all *p*s > .037, see Figure 20A).

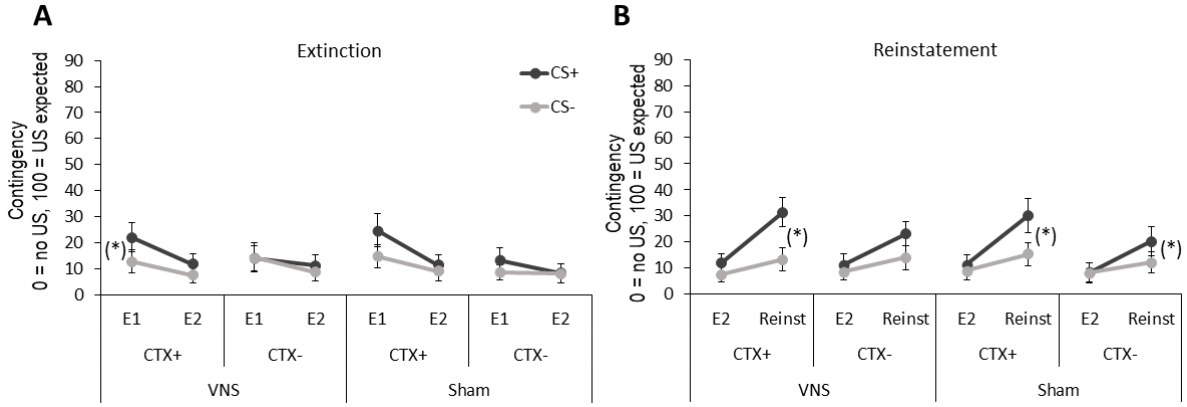

Figure 20: Contingency ratings of cues after extinction and reinstatement in Study 4.

Contingency ratings and standard errors (SE) for CS+ (black lines) and CS- (dark gray lines) are depicted separately for the anxiety context (CTX+, left) and the safety context (CTX-, right) for the tVNS group (left) and the sham group (right). (A) illustrates the ratings after the extinction phases 1 and 2 (E1, E2). (B) illustrates reinstatement effects by depicting contingency ratings after extinction (E2) and after reinstatement (Reinst). *: $p < .05$. Stars in brackets do not survive Bonferroni correction.

Reinstatement.

Startle. Comparing the last two startle responses between CS+ and CS- in CTX+ and CTX- during extinction for each group separately, no differences were found (all *p*s > .079). Therefore, the requirement for the calculation of reinstatement effects were met. In the tVNS group, CS+ elicited potentiated startle responses compared to CS- at the beginning of the test phase in CTX+ ($F(1,26) = 11.68$, $p = .002$, $\eta_p^2 = .310$) but not in CTX- ($F(1,26) = 3.26$, $p = .083$, $\eta_p^2 = .111$), which indicated differential reinstatement. The sham group showed a tendency for potentiated startle responses after reinstatement for CS+ compared to CS- in CTX+, however, statistics did not survive Bonferroni correction of $p < .006$ ($F(1,24) = 7.87$, $p = .010$, $\eta_p^2 = .247$). Contrasts between CS+ and CS- in CTX- returned no differences in sham stimulated participants ($F(1,24) = 0.16$, $p = .692$, $\eta_p^2 = .007$).

Ratings. Contrasts for CS+ and CS- after reinstatement demonstrated neither significant differences in CTX+ nor in CTX- in valence ratings (all *p*s > .132), arousal (all *p*s > .016, see Figure 19B), and in fear ratings (all *p*s > .080) irrespective of the group (Bonferroni-

corrected $p < .006$). A tendency of higher contingency ratings for CS+ compared to CS- was revealed in CTX+ and CTX- for both groups, however, statistics did not survive Bonferroni correction (all *p*s > .007, see Figure 20B).

Generalization.

Startle. Contrasts of startle responses revealed only potentiated startle magnitudes for CS+ compared to CS- in CTX+ in the sham group ($F(1,24) = 7.98$, $p = .009$, $\eta_p^2 = .249$). Other contrasts between CS+ and CS- were not significantly different, neither for CTX+, CTX- and GCTX in the tVNS group (all *p*s > .058), nor for CTX- and GCTX in the sham group (all *p*s > .279, Bonferroni-corrected $p < .008$, see Figure 21A).

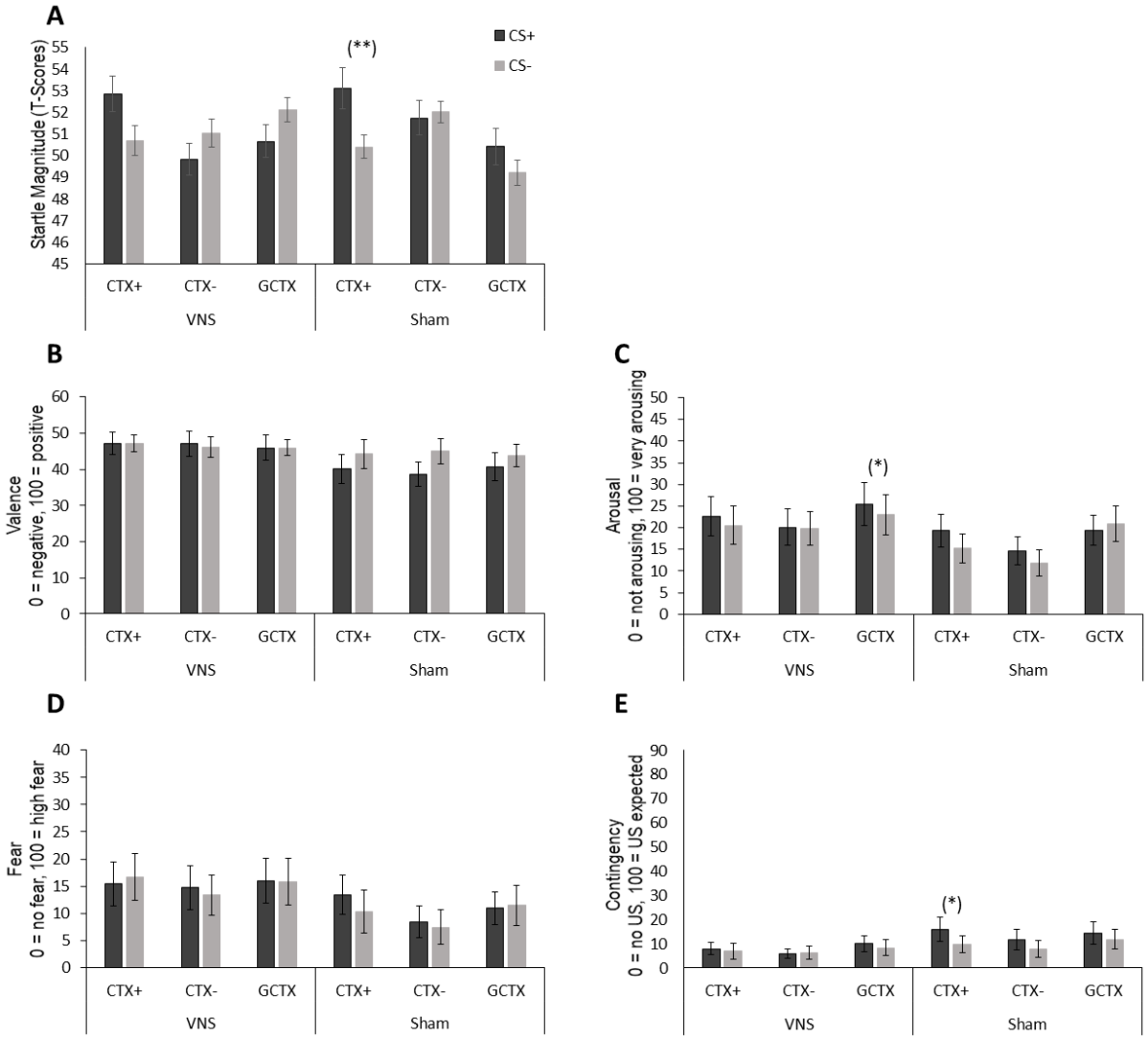

Figure 21: Startle responses and ratings of cues during generalization in Study 4.

Depicted are in (A) T-scores of startle responses with standard errors (SE) for CS+ (black bars) and CS- (gray bars) in the anxiety context (CTX+, left), in the safety context (CTX-, middle), in the generalization context (GCTX; right) for the tVNS group (left) and the sham group (right). (B), (C), (D), and (E) show valence, arousal, fear, and contingency ratings, respectively for CS+ and CS- separately for contexts and groups. *: $p < .05$; **: $p < .01$. Stars in brackets do not survive Bonferroni correction.

Ratings. No significant differences between CS+ and CS- in both contexts irrespective of group were found neither in valence (all *p*s > .055), nor in arousal (all *p*s > .048, Bonferroni-corrected significant $p < .008$), nor in fear ratings (all *p*s > .222) indicating robust subjective extinction learning without fear generalization (see Figure 21B-D). A trend towards increased arousal in CTX+ for CS+ compared to CS- was assessed only in the tVNS group ($F(1,26) = 4.27$, $p = .049$, $\eta_p^2 = .141$). Similar to the results of startle

responses, a trend of higher contingency ratings for CS+ compared to CS- in CTX+ was shown in the sham group ($F(1,24) = 6.47$, $p = .018$, $\eta_p^2 = .212$), but not in the tVNS group ($F(1,26) = 0.15$, $p = .700$, $\eta_p^2 = .006$; see Figure 21E). Other comparisons between CS+ and CS- did not return significant results, neither in the tVNS group (all *p*s > .498) nor in the sham group (all *p*s > .056).

5.3.5 Context Conditioning

Habituation.

The ratings of the contexts after the habituation phase revealed differences neither in valence, nor in arousal, nor in anxiety ratings (all *p*s > .447), which fulfilled the requirement of two similar contexts for the subsequent differential conditioning paradigm.

Acquisition.

Startle. As expected, the startle responses in A1 were potentiated in CTX+ compared to CTX- ($F(1,51) = 76.51$, $p < .001$, $\eta_p^2 = .600$) and compared to ITI ($F(1,51) = 14.11$, $p < .001$, $\eta_p^2 = .217$), which indicated context conditioning already during the first acquisition phase (see Figure 22A). The ITI also returned increased startle responses compared to CTX- ($F(1,51) = 21.75$, $p < .001$, $\eta_p^2 = .299$). A similar pattern revealed A2: Startle responses in CTX+ were potentiated compared to startles in CTX- ($F(1,51) = 23.35$, $p < .001$, $\eta_p^2 = .314$) and compared to startles during ITI ($F(1,51) = 8.39$, $p = .006$, $\eta_p^2 = .141$), which manifests successful context conditioning on physiological level. Startles during ITI were higher

compared to startles in CTX- ($F(1,51) = 11.84$, $p = .001$, $\eta_p^2 = .188$). After Bonferroni-correction $p < .008$ reached significance.

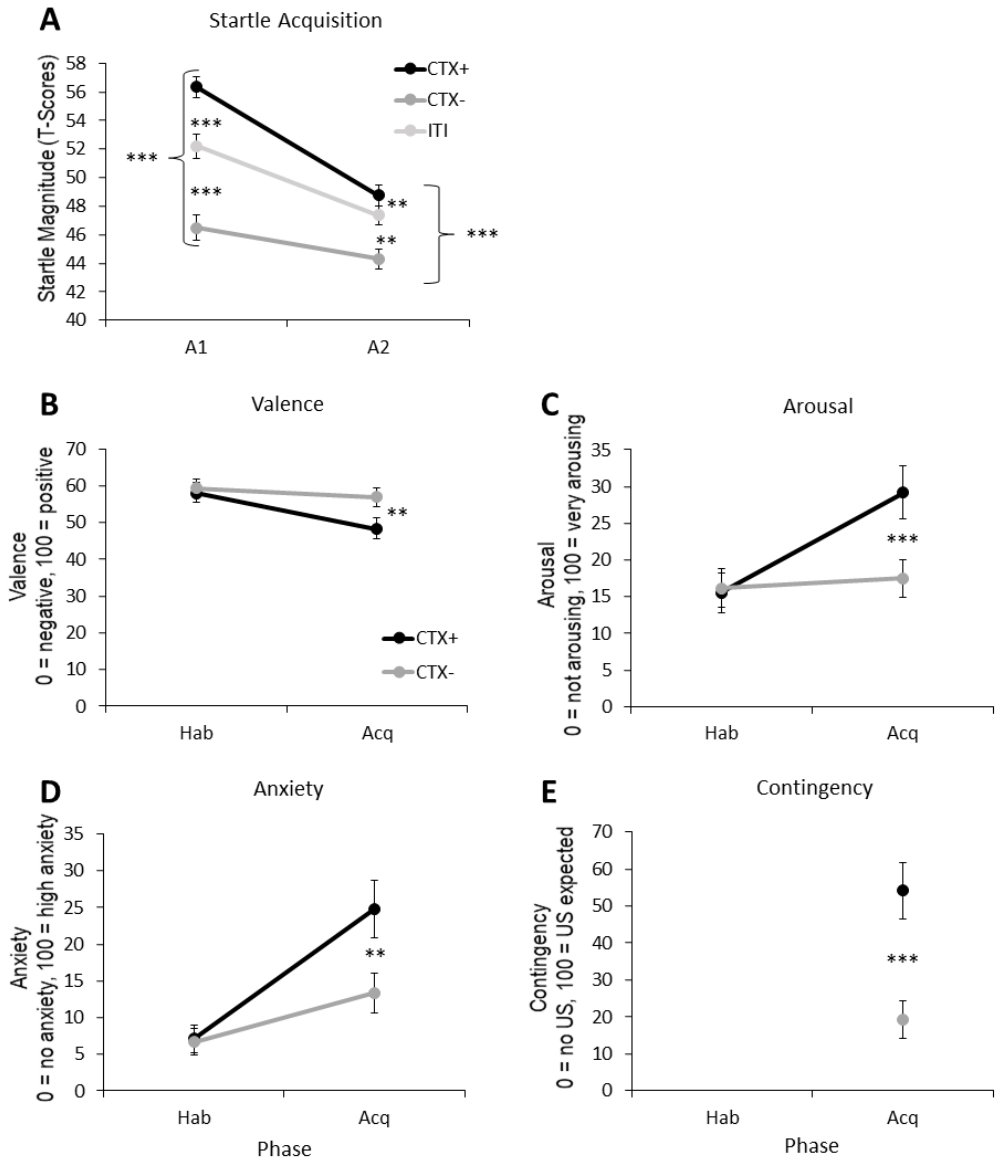

Figure 22: Context conditioning of Study 4.

Depicted are results of the startle response (A) for Acquisition 1 and 2 (A1, A2), valence (B), arousal (C), anxiety (D), and contingency ratings (E) after habituation (Hab) and after Acquisition (Acq) with standard error (SE). Black lines indicate the anxiety context (CTX+), dark gray lines the safety context (CTX-) and light gray lines the inter-trial interval (ITI). **: $p < .01$; ***: $p < .001$.

Ratings. More negative valence ratings for CTX+ vs. CTX- ($F(1,51) = 7.68$, $p = .008$, $\eta_p^2 = .131$) as well as higher arousal ($F(1,51) = 13.19$, $p = .001$, $\eta_p^2 = .206$), anxiety ($F(1,51) = 10.68$, $p = .002$, $\eta_p^2 = .173$) and contingency ratings ($F(1,51) = 42.24$, $p < .001$, $\eta_p^2 = .453$)

for CTX+ vs. CTX- indicated successful context conditioning on subjective level (Figure 22B-E).

Extinction.

Startle. The analyses of extinction in the tVNS group demonstrated no differences in startle responses during CTX+, CTX-, and ITI neither in E1 nor in E2 (all *p*s > .100, after Bonferroni-correction $p < .004$ reached significance), which suggests successful extinction learning. In the sham stimulated group, in E1 startle responses in CTX+ were higher compared to CTX- ($F(1,24) = 19.86$, $p < .001$, $\eta_p^2 = .453$) and also higher in ITI compared to CTX- ($F(1,24) = 17.79$, $p < .001$, $\eta_p^2 = .426$) signaling slow extinction. In E1, there was no difference between CTX+ and ITI ($F(1,24) < 1$). E2 revealed higher startle responses for ITI compared to CTX+ ($F(1,24) = 16.97$, $p < .001$, $\eta_p^2 = .414$) and compared to CTX- ($F(1,24) = 16.91$, $p < .001$, $\eta_p^2 = .413$). However, similar startle magnitudes for CTX+ and CTX- ($F(1,24) = 0.51$, $p = .481$, $\eta_p^2 = .021$) favor contextual extinction learning of the sham group during E2. The results are depicted in Figure 23 A and B.

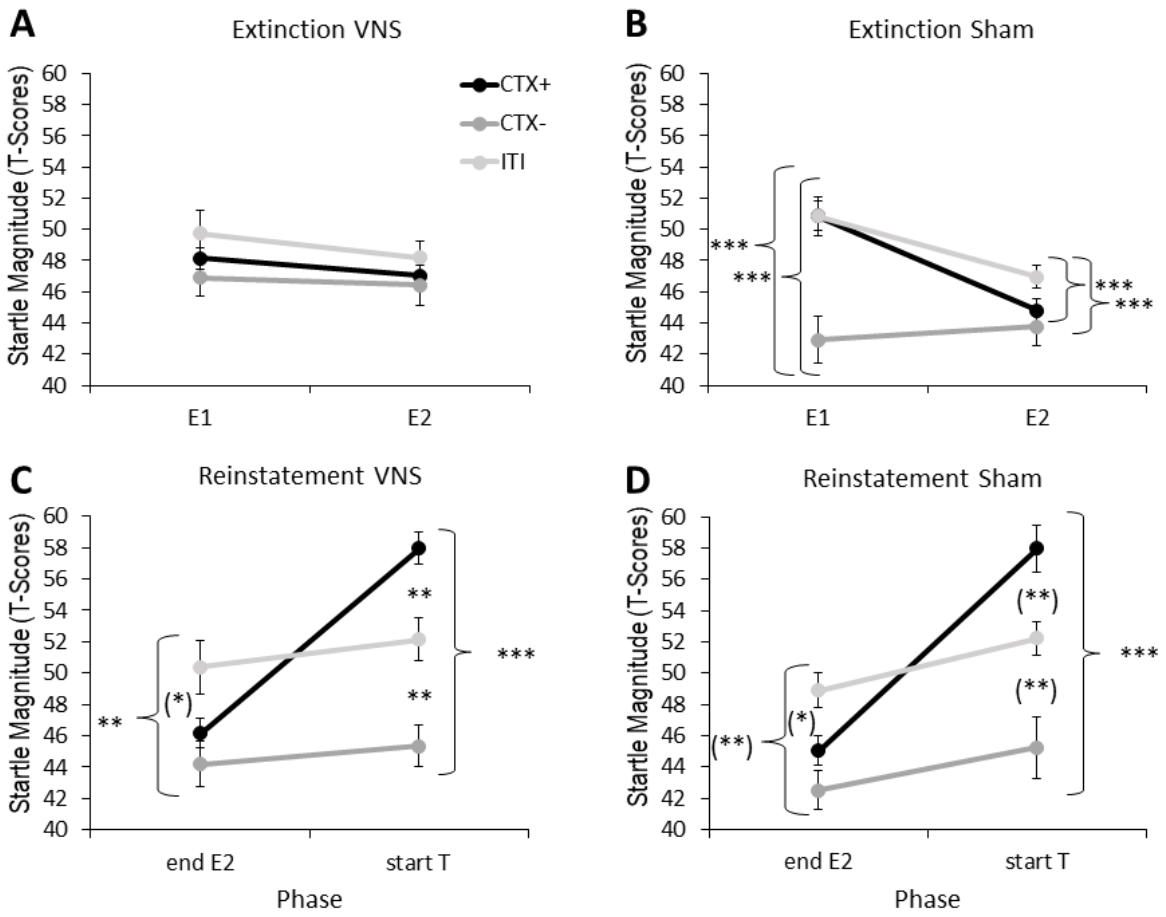

Figure 23: Startle responses during context extinction and reinstatement in Study 4.

On the upper panel, startle responses during extinction phases 1 (E1) and 2 (E2) are depicted for the tVNS group (A) and the sham group (B). The lower panel shows the last two startle responses in extinction (end E2) and the first two startle responses during test (start T) separated for the tVNS group (C) and the sham group (D). Standard errors (SE) are delineated. Black lines indicate the anxiety context (CTX+), dark gray lines the safety context (CTX-) and light gray lines the inter-trial interval (ITI). *: $p < .05$; **: $p < .01$; ***: $p < .001$. Stars in brackets do not survive Bonferroni correction.

Ratings. Successful extinction was also demonstrated in similar valence ratings of CTX+ and CTX- after E1 and after E2 in both tVNS and sham group (all *p*s > .150, Bonferroni-corrected $p < .004$). Arousal ratings differed between groups (see Figure 24 A and B). While in the tVNS group, participants rated CTX+ and CTX- similarly arousing after E1 and after E2 (all *p*s > .628), in the sham group, participants indicated higher arousal in CTX+ compared to CTX- after E1 ($F(1,24) = 5.72$, $p = .025$, $\eta_p^2 = .192$) and after E2 ($F(1,24) = 6.63$, $p = .017$, $\eta_p^2 = .216$), which indicates no extinction in the sham group. Similarly,

anxiety ratings in the tVNS group revealed no differences between contexts neither after E1 nor after E2 (all *p*s > .163).

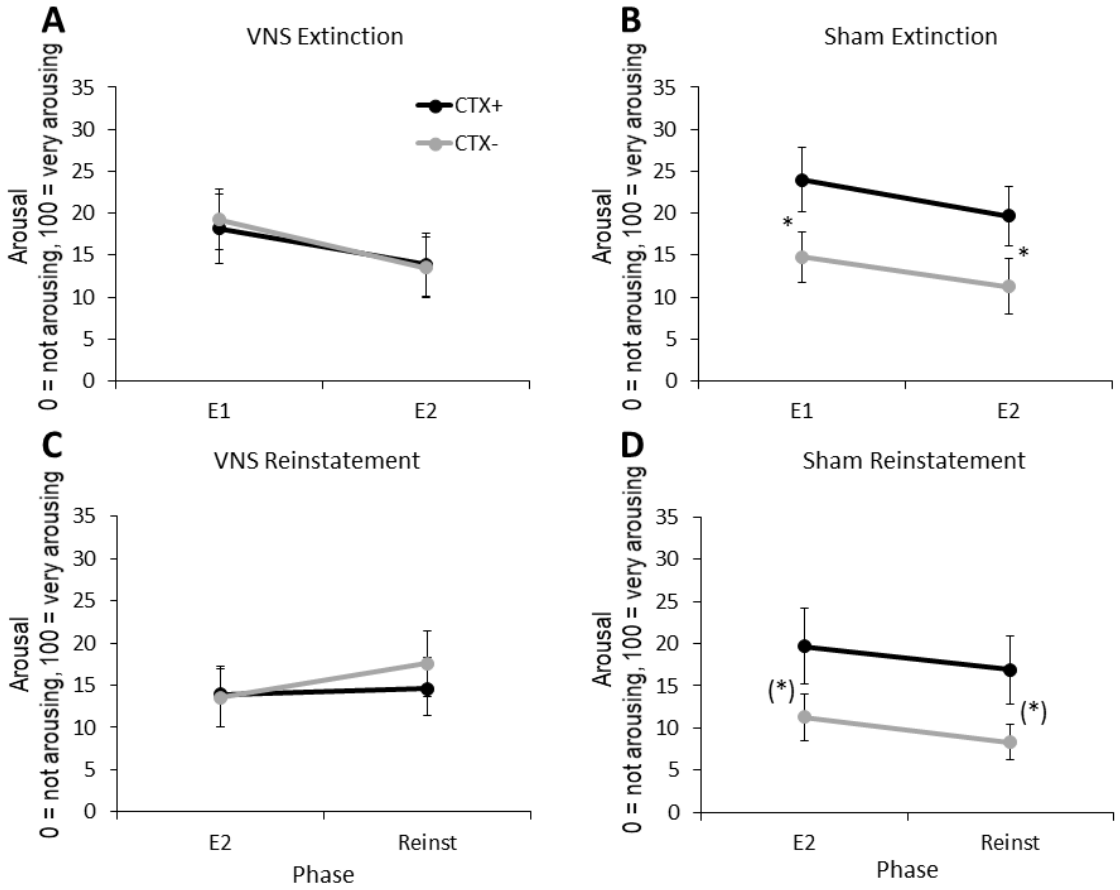

Figure 24: Arousal ratings of contexts after extinction and reinstatement in Study 4.

On the upper panel, arousal ratings are depicted after extinction 1 (E1) and 2 (E2) for the tVNS group (A) and the sham group (B). The lower panel compares arousal ratings after E2 with ratings after reinstatement (Reinst) for the tVNS group (C) and the sham group (D). Standard errors are delineated. Black lines indicate the anxiety context (CTX+), dark gray lines the safety context (CTX-). *: $p < .05$. Stars in brackets do not survive Bonferroni correction.

Interestingly, anxiety ratings in the sham group did also not differ between contexts, neither after E1 ($F(1,24) = 3.08$, $p = .092$, $\eta_p^2 = .114$) nor after E2 ($F(1,24) = 4.22$, $p = .051$, $\eta_p^2 = .149$) though they descriptively resemble the respective arousal ratings. In line with the reported results above, contingency ratings indicated similar US expectancy in CTX+ and CTX- in both groups after the first and second extinction phase (all *p*s > .072; see

Figure 25 A and B). The results suggest successful overall extinction in both groups, however, no extinction was measured in arousal of the sham group.

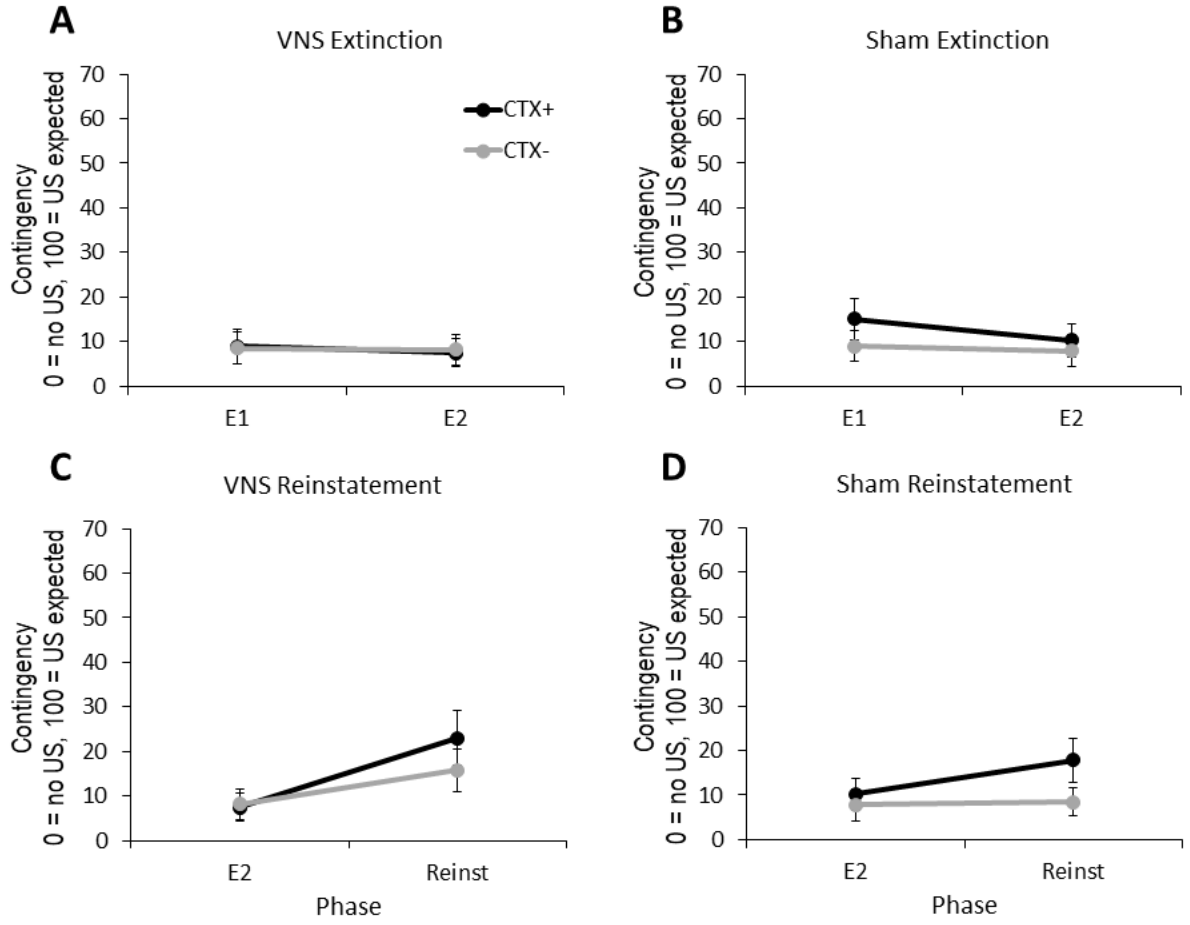

Figure 25: Contingency ratings of contexts after extinction and reinstatement.

On the upper panel, contingency ratings are depicted after Extinction 1 (E1) and 2 (E2) for the tVNS group (A) and the sham group (B). The lower panel compares contingency ratings after E2 with ratings after reinstatement (Reinst) for the tVNS group (C) and the sham group (D). Standard errors are delineated. Black lines indicate the anxiety context (CTX+), dark gray lines the safety context (CTX-).

Reinstatement.

Startle. For the tVNS group, contrasts between the last two startles during extinction returned higher startle responses for ITI compared to CTX- ($F(1,26) = 9.33$, $p = .005$, $\eta_p^2 = .264$), but no differences between CTX+ vs. CTX- and CTX+ vs. ITI (all $ps > .017$, see Figure 23 C and D). Interestingly, the first two startles in the test phase were higher in CTX+ compared to CTX- ($F(1,26) = 53.21$, $p < .001$, $\eta_p^2 = .672$) and compared to ITI ($F(1,26) =$

14.76, p = .001, η_p^2 = .362) and additionally for startles in ITI compared to CTX- (F(1,26) = 15.79, p = .001, η_p^2 = .178). Similarly in the sham group, startle responses in CTX+ and CTX- as well as CTX+ and ITI did not differ in late extinction (all ps > .012), but startle responses during the ITI were significantly potentiated compared to CTX- (F(1,24) = 11.29, p = .003, η_p^2 = .320). In the early test phase, startles in CTX+ were higher compared to CTX- (F(1,24) = 28.98, p < .001, η_p^2 = .547). After Bonferroni correction (p < .004), no difference was revealed neither between CTX+ and ITI nor between CTX- and ITI (all ps = .006). In sum, tVNS as well as sham group showed no difference in startle response between CTX+ and CTX- at the end of extinction, but after reinstatement, which indicates differential reinstatement.

Ratings. Valence ratings showed reinstatement neither in the tVNS nor in the sham group, which was indicated by similar ratings of CTX+ and CTX- after reinstatement (all ps > .402). In line, after Bonferroni corrections (p < .004) arousal ratings of CTX+ and CTX- after reinstatement differed neither in the tVNS nor in the sham group (all ps > .023, see Figure 24 C and D). Reinstatement effects of anxiety ratings were also absent in the tVNS as well as in the sham group: both contexts were rated as similar anxiogenic after reinstatement (all ps > .054). For both groups, contingency ratings for CTX+ vs. CTX- did not differ after reinstatement (all ps > .057). No other contrasts of contingency ratings of contexts after E2 and after reinstatement did significantly differ after Bonferroni corrections (all ps > .018; see Figure 25 C and D). Altogether, reinstatement effects were very low in both tVNS and sham group and differed barely. However, only contingency ratings in the tVNS group revealed increased US expectancy in CTX+ after reinstatement.

Generalization

Startle. Startle responses in the tVNS group were reduced in GCTX compared to CTX+ ($F(1,26) = 14.47$, $p = .001$, $\eta_p^2 = .357$) and compared to CTX- ($F(1,26) = 22.50$, $p < .001$, $\eta_p^2 = .464$), but did not differ between CTX+ and CTX- ($F(1,26) = 0.45$, $p = .507$, $\eta_p^2 = .017$; see Figure 26). In the sham group, startle responses for GCTX were decreased only in comparison with CTX+ ($F(1,24) = 12.95$, $p = .001$, $\eta_p^2 = .351$). Startles in CTX+ and CTX- as well as CTX- and GCTX elicited similar startle magnitudes (all *p*s > .078, Bonferroni-corrected $p < .017$).

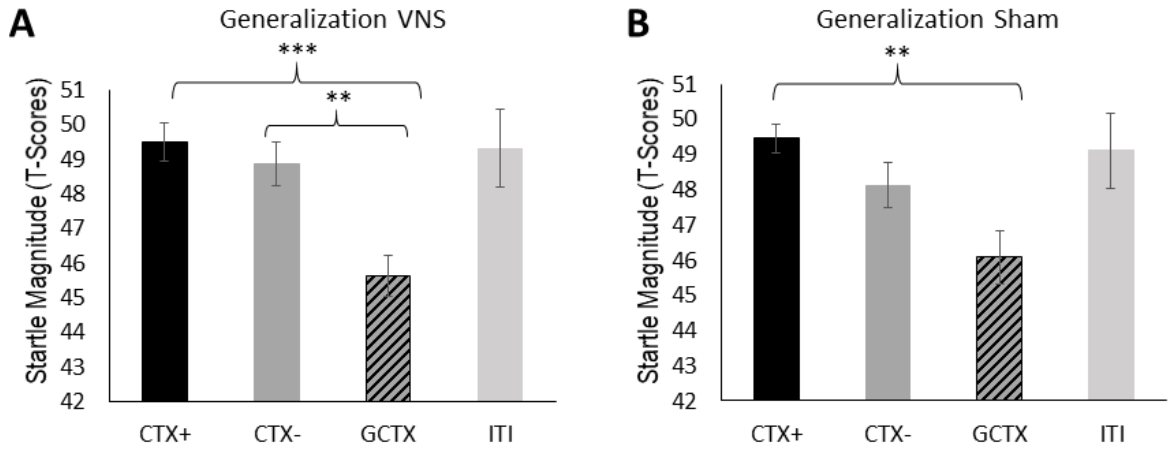

Figure 26: Startle responses during context generalization in Study 4.

(A) shows startle magnitudes of the tVNS group and (B) of the sham group. Depicted are T-scores of startle responses with standard errors (SE) in the anxiety context (CTX+, black bar), in the safety context (CTX-, dark gray bar), in the generalization context (GCTX; striped bar) and for comparison in the inter-stimulus interval (ITI, light gray bar). **: $p < .01$; ***: $p < .001$.

Ratings. Calculated contrasts (Bonferroni-corrected $p < .017$) of valence ratings revealed no differences between contexts neither in the tVNS nor in the sham group (all *p*s > .075). Arousal ratings of the tVNS group revealed similar contrasts between contexts (all *p*s > .057). Interestingly, the sham group rated CTX+ as higher arousing compared to CTX- ($F(1,24) = 7.03$, $p = .014$, $\eta_p^2 = .227$) and GCTX higher arousing compared to CTX- ($F(1,24) = 6.82$, $p = .015$, $\eta_p^2 = .221$). Arousal ratings of CTX+ and GCTX did not differ

($F(1,24) = 0.09$, $p = .763$, $\eta_p^2 = .004$). Anxiety and contingency ratings did not differ between contexts during generalization, neither in the tVNS nor in the sham group (all *p*s > .038), which is depicted in Figure 27.

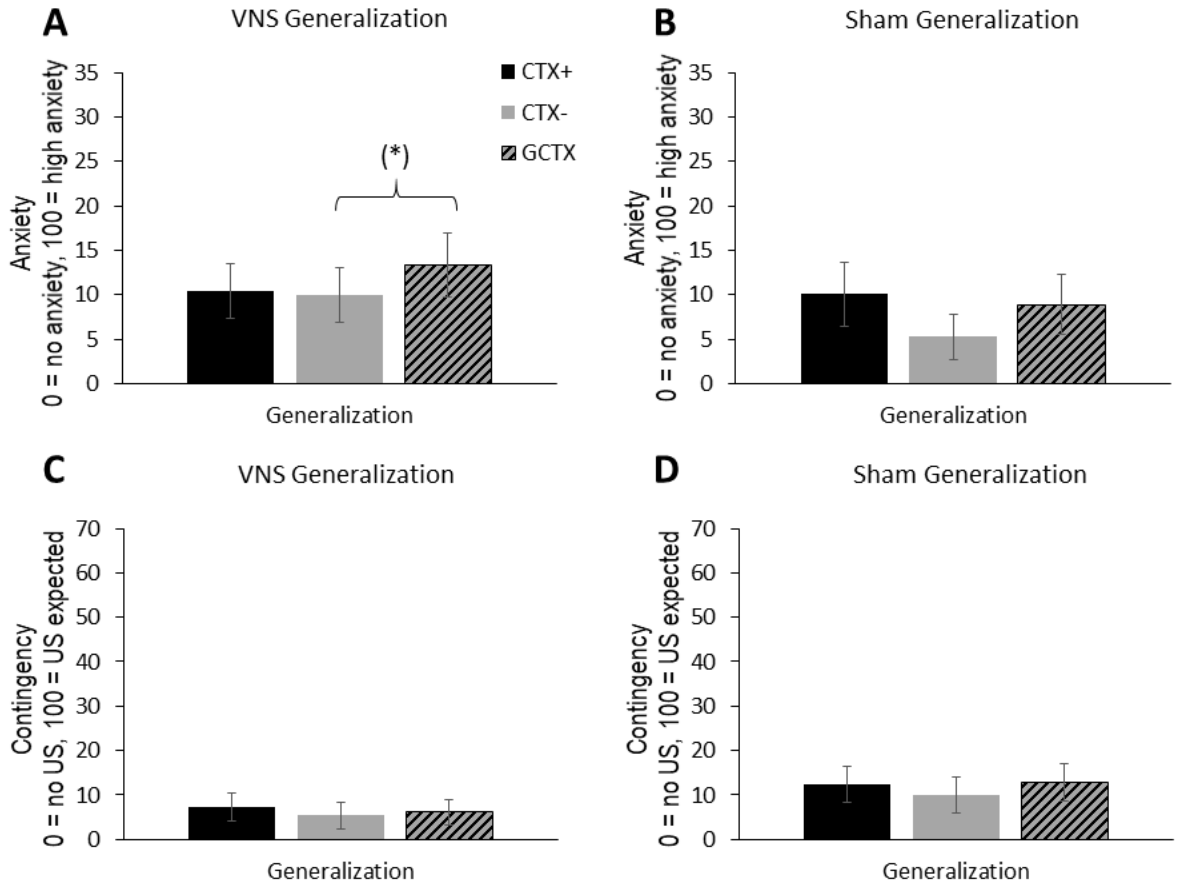

Figure 27: Anxiety and contingency ratings of context generalization in Study 4.

Depicted are anxiety ratings with standard error (SE; upper panel) from the tVNS group (A) and the sham group (B) and contingency ratings with standard error (SE; lower panel) from the tVNS group (C) and from the sham group (D) after generalization. The black bar indicates the anxiety context (CTX+), the dark gray bar indicates the safety context (CTX-), and the striped bar indicates the generalization context (GCTX). *: $p < .05$. Stars in brackets do not survive Bonferroni correction.

5.3.6 Exploratory Analyses

State anxiety and reinstatement. In parallel to the exploratory analyses of Study 2, we here calculated the median split of participants' STAI state score on Day 2 before reinstatement (split value was 34) resulting in 26 low anxious participants ($M = 27.50$, $SD = 3.82$) and 26 high anxious participants ($M = 40.65$, $SD = 7.56$). In order to investigate the

effects of state anxiety on reinstatement in the startle response, we calculated a 3 x 2 x 2 ANOVA with the within-subject factors context (CTX+, CTX-, ITI) and phase (end E, start T) and the between-subjects factor state anxiety (low anxious, high anxious). Significant main effects of phase ($F(1,50) = 39.10$, $p < .001$, $\eta_p^2 = .439$) and context ($F(2,100) = 49.05$, $p < .001$, $\eta_p^2 = .495$) and the interaction of Phase x Context were found ($F(2,100) = 18.48$, $p < .001$, $\eta_p^2 = .270$). However, neither main effect of state anxiety, nor interactions were revealed (all *p*s > .262). In sum, we could not find any influence of state anxiety on reinstatement effects in the context conditioning part of this context-dependent cue conditioning paradigm.

Early generalization. The reported results during both context and context-dependent cue generalization demonstrated very stable extinction learning and therefore slight generalization effects even after reinstatement. Another explanation might be the relatively long generalization phase including 2 presentations of each context without any US. The low ratings afterwards might be caused by bottom effects. To avoid this, we separately investigated generalization effects in the first two startle responses during generalization. For contextual generalization, we calculated a repeated measures ANOVA with the within-subject factors context (CTX+, CTX-, GCTX) and the between-subject factor group (tVNS, sham). A significant main effect of context ($F(2,100) = 20.88$, $p < .001$, $\eta_p^2 = .295$) was revealed, but no main effect of group and no interaction of Context x Group (all *p*s > .579). Following up the main effect with post-hoc *t*-tests revealed significantly increased startle responses in CTX+ and CTX- compared to GCTX ($t(51) = 5.92$, $p < .001$; $t(51) = 6.40$, $p < .001$, respectively) but no difference between CTX+ and CTX- ($t(51) = 0.17$, $p = .865$). For context-dependent cue conditioning, we extended the ANOVA by the within-subject factor cue (CS+, CS-). The significant main effect of context ($F(2,100) = 3.83$, $p = .025$, $\eta_p^2 = .071$) and the significant interaction of Context x Cue ($F(2,100) = 13.11$,

$p < .001$, $\eta_p^2 = .208$) were found. However, no further main effects and interactions were revealed (all *p*s > .138). Following up the interaction effect, post-hoc *t*-tests revealed significantly higher startle responses for CS+ compared to CS- in CTX+ ($t(51) = 4.44$, $p < .001$). However, no differences were found in CTX- and GCTX (all *p*s > .112), indicating no fear generalization in participants independent of the kind of stimulation they got during extinction. One can conclude successful and stable extinction learning on physiological level.

5.4 Discussion

The main goals of the current study were first, the investigation of more effective transcutaneous vagus nerve stimulation parameters and its implementation; and second, the use of contexts as background for context-dependent cue conditioning, reinstatement, and additional generalization. Therefore, I prolonged the overall stimulation time, added sham stimulation to the acquisition and test phase, and adapted the VR paradigm to investigate tVNS effects on context as well as on cue conditioning.

Context-dependent cue conditioning was only successful in valence ratings, indicated by lower valence for CS+ compared to CS- in CTX+, but not in CTX- after the acquisition. In fact, CS+ elicited potentiated startle responses and higher arousal, fear, and contingency ratings compared to CS- independent of context, and therefore, independent of the actual pairing of the CS+ with an US during acquisition. Despite the unsuccessful context-dependent cue conditioning, startle responses, as well as all ratings, revealed successful extinction indicated by similar responses to CS+ and CS- during or after extinction. Interestingly, I assessed differential reinstatement regarding potentiated startle responses for CS+ compared to CS- in both groups only in the anxiety but not in the safety context, which required some kind of physiological context-dependent cue learning

during acquisition. No reinstatement was found in ratings, which again signals very stable extinction. However, contingency ratings demonstrated slightly increased US expectancy for CS+ compared to CS- in both contexts independent of the stimulation group. During generalization, only the sham group showed a trend towards increased startle magnitudes for CS+ compared to CS- in the anxiety context, but neither in the safety nor in the generalization context, whereas CS+ vs. CS- comparisons did not return significant differences in the tVNS group. Subjective ratings did not differ between CS+ and CS-.

Though the experimental design was slightly changed compared to pure context conditioning in Study 2, I found context conditioning on physiological as well as subjective level indicated by potentiated startle responses, lower valence, and higher arousal, anxiety, and contingency ratings in CTX+ compared to CTX- during or after the acquisition. Interestingly, successful context extinction was revealed in the tVNS group for all dependent variables and in the sham group for valence, arousal and contingency ratings, whereas incomplete context extinction was assessed in the sham group regarding startle responses and arousal ratings. For both groups, differential context reinstatement was found in startle responses rather than generalized reinstatement, which was expected from Study 2. On the subjective level, no return of anxiety was found. Assuming a more stable extinction in the tVNS group (Burger et al., 2016; Peña et al., 2013), for the generalization test I expected anxiety responses in GCTX that were similar to CTX- in the tVNS group and similar to CTX+ in the sham group. However, startle responses in GCTX were similar to CTX- in the sham group, and even significantly lower in the tVNS group. The robust extinction, which was on a subjective level even stable to reinstatement, might have been the reason for no differentiation of the contexts during generalization and overall very low anxiety ratings in both groups.

In parallel to Study 2 (Genheimer et al., 2017), I monitored heart rate changes during extinction as a result of vagus nerve stimulation. The lack of a main effect of group indicated either no changes in HR induced by vagus nerve stimulation in comparison to the sham group, or a slight stimulation in both groups according to the inverted U-shape of optimal vagal stimulation (Clark, Krahl, Smith & Jensen, 1995; Clark et al., 1998). Thereby, I speculate that stimulation of the helix could slightly have activated the vagus nerve because of tingling sensation ranging over the outer ear. Regarding the inverted U-shaped stimulation curve (Clark et al., 1995; Clark et al., 1998), sham stimulation might have increased vagal activity by very low stimulation, whereas the relatively high intensity of verum tVNS might also slightly have activated the vagus nerve, but from the other side of the inverted U-shaped curve. When the HR data are interpreted regarding the predator imminence model (Fanselow, 1994), lower HR at the beginning of the extinction phase in CTX+ could indicate an anxiety response by orientation reaction towards potential threat in the surrounding. In the second extinction phase, i.e. extinction Trials 3 and 4, participants might have expected a change in CTX–US contingencies and therefore showed higher orienting responses, i.e. decelerated HR, in CTX- compared to CTX+. Altogether, the HR analyses were insufficient for a reliable manipulation check of tVNS, but indicated comprehensible anxiety responses according to the predator imminence model.

In sum, I revealed weak context-dependent cue learning, which emphasizes the important role of context during fear acquisition and pronounced overall context conditioning. Moreover, the changes of vagus nerve stimulation parameters were still insufficient for clear evidence for extinction accelerating and stabilizing effects in humans.

Except for valence ratings, dependent variables showed increased responses to CS+, independent of the context in which it was presented. One could argue that the study

design without any learning instructions was too complicated to learn in only few trials. Alternatively, the cue stimuli, i.e. the colored lights, might have increased the ambiguity of the safety context. Stimulus generalization of CS+ and CS- might have caused such results. However, Andreatta et al. (submitted-a) and Mühlberger et al. (2014) used similar paradigms and reported successful context-dependent cue conditioning regarding fear-potentiated startle, increased skin conductance response as well as higher fear ratings for CS+ compared to CS- in CTX+ but not in CTX-. Though the paradigm and the methodology during acquisition were very similar, one difference between the current study and Andreatta et al. (submitted-a) as well as Mühlberger et al. (2014) was the technology to present the VR. Whilst Andreatta et al. (submitted-a) and Mühlberger et al. (2014) used a head-mounted display (HMD), I used the Powerwall to present the stimuli. One could argue that the HMD blocks participants' attention off from the laboratory context towards VR itself, in which the cues become foreground stimuli and attention was focused easily. Moreover, the participant's real or virtual body was not depicted. In contrast, the Powerwall provides a more context-specific presentation of VR due to the participant sitting about 1.5 m in front of the huge Powerwall wearing 3D glasses but still recognizing the space between themselves and the VR including their own body in the specific context. In the current experiment, this could have resulted in higher saliency of the context rather than the cues and therefore could have reduced the context-dependent cue learning but reinforced the context learning itself. One common advantage of all three described context-dependent cue conditioning studies is the VR, which provides the possibility to use cues that could naturally be an element of the context (Maren et al., 2013) rather than e.g. geometric shapes (see Haaker et al., 2013; Marschner et al., 2008), which are artificially placed above a background context but do not belong to the context and can

therefore not be represented conjunctively in the brain (Nadel & Willner, 1980; Rudy, 2009; Rudy et al., 2004).

During the extinction phase, all participants extinguished their fear of CS+. Surprisingly, extinction learning for the cues seemed to be slower in the tVNS group, which showed still potentiated startle responses for CS+ compared to CS- during the first extinction phase, but not during the second. This contrasts with the slower contextual extinction in the sham group. Indeed, according to Çalışkan and Albrecht (2013), VNS leads to NE release and therefore neural plasticity in the amygdala, hippocampus and mPFC, which are associated with successful extinction learning (Milad & Quirk, 2002; VanElzakker et al., 2014). Based on those findings, the combination of extinction learning and increased neural plasticity by tVNS should strengthen extinction learning. However, especially context conditioning depends on the hippocampal activity. An imaging study by Frangos et al. (2015) found deactivation of the hippocampus during vagus nerve stimulation. Their analysis over stimulation time showed a change in activity in the hippocampal activity. As contextual extinction relies on the hippocampus, I speculate that tVNS might have changed hippocampal activity during extinction, which resulted in faster contextual extinction learning in the tVNS compared to the sham group. However, the slower cue extinction in the tVNS group regarding the startle response stays unclear. One explanation might be the unspecific fear acquisition, which resulted in generalized fear response to CS+ independent of the context and therefore in unexpected extinction learning. Future studies should try to disentangle the brain areas, that were effectively activated by the combination of tVNS and cue and context extinction in humans, e.g. by simultaneous fMRI.

Physiological reinstatement of fear revealed generalized reinstatement for CS+ and CS- in the safety context, and differential reinstatement, i.e. potentiated startle responses for

CS+ compared to CS- only in the anxiety context, whereby the effect was more pronounced in the tVNS compared to the sham group. In contrast, I expected more stable extinction memory in the tVNS group and therefore fewer reinstatement effects also on cued reinstatement. One reason for such differences could be the stimulation method. While the sham group got sham stimulation at the helix of the ear during each phase of the experiment, the tVNS group was sham stimulated during acquisition and reinstatement and vagus nerve stimulated at the cymba conchae of the ear during extinction. Though participants did not report consciously observed stimulation differences or alterations, this slight change of the context during extinction could still be the reason for the higher return of fear due to the reminder of the acquisition context in the tVNS group during reinstatement. As reported above, several studies on return of fear investigated renewal using ABA designs, meaning acquisition in context A, extinction in context B, and test in context A and reported a recovery of the fear regarding fear-potentiated startle, skin conductance, fear, as well as US expectancy ratings (Alvarez, Johnson & Grillon, 2007; Bouton, 2002; Bouton & Bolles, 1979; Vansteenwegen, 2005; Vervliet, 2013). Therefore, a slight contextual change during extinction only in the tVNS group might have deteriorated extinction learning. As this effect overlaps with the contrasting effect of tVNS, both could have compromised extinction learning in the tVNS group. Besides a trend in contingency ratings, no subjective reinstatement could be found neither in the tVNS nor in the sham group. The study design might be the reason for this. Fear acquisition and extinction were performed on the same day, separated by only 30 min of the stimulation phase. Hence, the newly learned CS+–US association during acquisition was immediately contrasted by the inhibitory CS+–noUS association of the extinction memory (see e.g. Vervliet, 2013). As overnight memory consolidation strengthens a newly acquired memory (Diekelmann & Born, 2010; Menz et al., 2013), and the extinction memory was

the latter memory trace built on Day 1, the CS+–noUS association might be stronger and surpass the fear memory resulting in a lack of reinstatement.

The generalization results of context-dependent cue conditioning revealed no differences between CS+ and CS-, neither in anxiety nor in safety nor generalization context. One reason for this could be the unspecific fear acquisition on Day 1. Increased fear responses to all CS+ independent of context during acquisition supports the idea of generalized fear acquisition rather than distinct context-dependent cue learning. Though extinction was successfully performed, the lack of the previously created context-dependent fear memory does not enable the investigation of cue generalization. Furthermore, the robust extinction memory and even the low reinstatement effects indicate a weak present acquisition memory and therefore no possible cue differentiation in various contexts by the recall of the acquisition memory. In a side project, Burger et al. (2017, see supplementary material) did not find tVNS effects on stimulus generalization, though faster extinction was revealed in US expectancy ratings. A current study by Burger et al. (2019) specifically investigated the effects of tVNS on generalization and used a more classical approach of fear conditioning (Lissek et al., 2014) directly followed by a generalization phase and additional subsequent extinction. Though they replicated their prior results on decreased US expectancy ratings by tVNS during extinction, they reported no tVNS effects on generalization, neither in startle response nor in US expectancy ratings. They argue first, that their conditioned and generalization stimuli, i.e. circles in different sizes, may not have been discriminated by the participants (Burger et al., 2019). Second, during generalization, the CS+ was still reinforced in 50% of the presentations, which resulted in an ambiguous, still threatening situation, in which it might have been adaptive to respond with fear independent of the presented stimulus (Burger et al., 2019). Altogether, Burger et al. (2017) and (2019) and the current study are the only studies,

which have investigated tVNS effects on fear generalization. Therefore, more research is needed, especially by using more reliable study designs and better stimulus control.

Here, I used a more complex paradigm than in Study 2 but still could demonstrate appropriate context conditioning on physiological as well as on the subjective level. In fact, I replicated results of earlier context conditioning studies either by using VR (Andreatta et al., 2015a; Genheimer et al., 2017; Glotzbach-Schoon et al., 2013a) or by prolonged single stimulus presentation (Pohlack et al., 2012; Vansteenwegen, 2005). Interestingly, mixed results of context conditioning in context-dependent cue conditioning paradigms have been found in previous studies. Baas et al. (2004) described fear-potentiated startle responses in CTX+ compared to CTX-, Huff et al. (2011) reported increased SCR in CTX+ compared to CTX- especially at the beginning of acquisition, and Marschner et al. (2008) emphasized hippocampal activity particularly in the unpredictable US conditions of their experiment as well as early in acquisition when contingencies are more unpredictable. Moreover, Mühlberger et al. (2014) found increased startle responses and slightly increased anxiety ratings for CTX+ compared to CTX- during acquisition, however, they also report interactions of Context x Cue indicating context-dependent cue learning. In contrast, in their very similar study design, Andreatta et al. (submitted-a) unexpectedly found increased startle responses in CTX- compared to CTX+, which could be explained by the contexts serving as background stimuli for the cue learning. However, in all studies described above, only the combination of the anxiety context and CS+ reliably predicted the US, which still suggests higher physiological activity in the anxiety context. Overall, our successful context conditioning results are in line with the majority of previous literature and even expand the investigations of successful context conditioning by valence, arousal and contingency ratings after the

acquisition and additionally emphasize the important role of contexts in fear conditioning experiments.

Interestingly, the effects of successful vagus nerve stimulation could be revealed in the extinction of contextual anxiety. Namely, the tVNS group showed similar startle responses as well as similar ratings for CTX+ and CTX- already after the first extinction phase. In contrast, the sham group showed differences in context perception in startle responses during the first extinction phase, which disappeared in the second extinction phase indicating successful but slower extinction learning. Extinction in arousal ratings even turned out to be incomplete in the sham group. These results are in accordance to my hypothesis and previous conditioning studies in animals (Peña et al., 2014; Peña et al., 2013) and humans (Burger et al., 2017; Burger et al., 2016) and show accelerated extinction learning by vagus nerve stimulation. One reason for such context-related effects could be due to a facilitated communication between the amygdala and hippocampus in a cue-in context conditioning paradigm because of the interaction of contexts and cues (Alvarez et al., 2008; Andreatta et al., 2015a; Marschner et al., 2008). The adaptions of stimulation parameters, i.e. prolonged stimulation time pre-extinction and between the two extinction phases could have been another reason for such group differences in our current but not in our previous context conditioning study (Genheimer et al., 2017). In contrast to other studies, which did not find tVNS effects on extinction in physiological measures (Burger et al., 2017; Genheimer et al., 2017), I here elaborate the first evidence of tVNS effects for faster extinction learning also on the physiological level, i.e. startle response.

Based on animal studies (Peña et al., 2014; Peña et al., 2013), I expected tVNS causing more stable extinction memory concerning the return of fear and anxiety. However, differential reinstatement of startle responses was similar for tVNS and sham group. This

contradicts the generalized return of anxiety in startle responses in our previous study (Genheimer et al., 2017). Interestingly, Glotzbach-Schoon et al. (2015) reported differential reinstatement in high state anxious participants and generalized reinstatement in low anxious participants, calculated with a median split of the STAI state questionnaire (Laux et al., 1981; Spielberger et al., 1970) resulting in a mean of 36.20 (*SD* = 10.14) for those participants who received reinstatement. Comparing the results of the STAI state questionnaires (Laux et al., 1981; Spielberger et al., 1970) in Study 2 (*M* = 34.47; *SD* = 7.30) and current study (*M* = 34.07; *SD* = 8.90), the scores are very similar and lower than in Glotzbach-Schoon et al. (2015) arguing for generalized reinstatement. Alternatively, the study designs and learning paradigms rather than state anxiety might modulate the kind of reinstatement. In Genheimer et al. (2017) a pure context conditioning paradigm with a US reinforcement rate of 75% during acquisition revealed generalized reinstatement in startle response indicating a similar return of anxiety in both office contexts, though only one has previously been associated with the aversive US. Hence, the similarity of anxiety and safety context could be one reason for the generalization of reinstatement, indicating first the ambiguity of a formerly safety situation, namely the safety context, and second demonstrating a 'better safe than sorry' survival strategy. Interestingly, in the current study, the context alone was no predictor for an US, but only the combination of anxiety context with CS+ predicted the US in the acquisition phase in 100% of the trials, which required a more elaborate learning process. Though the anxiety context alone has never been directly paired with an US, the absence of any other cue during context presentation might have boosted differential reinstatement. Unexpectedly, I only found differential reinstatement in contingency ratings for the tVNS but not for the sham group, which could either indicate differential recall of previously learned and consolidated anxiety and safety contexts in the tVNS

group, or lower extinction learning than the sham group resulting in a weaker explicit extinction memory and higher return of anxiety. No other subjective reports revealed evidence for reinstatement. One reason could be the very stable extinction learning of contextual anxiety. An alternative explanation might be the splitting of the study in two consecutive days, whereby acquisition and extinction took place in one day, reinstatement and generalization however, on the second day. The anxiety memory trace is built up during acquisition, but in a similar way, the extinction memory trace as well (Bouton, 2002). The competition of both traces and the latter extinction phase might have weakened the anxiety memory trace. This could have resulted in less anxiety memory consolidation, which explains the stable extinction and therefore the lack of subjective reinstatement effects (Bouton, 2002; Milad & Quirk, 2012; Quirk, 2002; Quirk & Mueller, 2008). In fact, during context reinstatement, not only the context i.e. the black screen, in which the three USs were delivered, changed compared to the acquisition on Day 1, but likely the context of participants' attitude towards the experiment and their internal state or motivation might have changed on Day 2. In their review, Haaker et al. (2014b) summarized various conditioning studies that partly found generalized and differential reinstatement. They propose the study designs as a relevant factor for the investigation of reinstatement. On the one hand, the discrimination of safety cues or contexts in potentially threatening situations reduces the risk of pathological anxiety (Lissek et al., 2005). On the other hand, the maintenance of this discrimination in threatening situations could result in remission of fear or anxiety in the long run (Haaker et al., 2014b). In differential conditioning protocols, the conditioned stimuli are very similar. Therefore, generalized reinstatement might be caused by associative learning processes and stimulus generalization of CS+ and CS- (Haaker et al., 2014b). Alternatively, generalized reinstatement might be due to orientation to ambiguous stimuli or contexts, which is

given after reinstatement (Haaker et al., 2014b). Haaker et al. (2014b) suggested to address this issue by either adding a control group without reinstatement (e.g. Glotzbach-Schoon et al., 2015) or by adding control stimuli, that are only present in the acquisition phase or reinstatement test, which would control for associative learning (e.g. Mühlberger et al., 2014).

Overall, the responses to the generalization context were very low, which is likely due to bottom effects because of the extinction phase in between acquisition and generalization phases. As such, the tVNS group showed lower startle responses to GCTX compared to anxiety and safety context, which indicated no generalization at all. Even further, this could be interpreted as safety responses to GCTX versus CTX+ and CTX-, which were previously either directly associated with an US or at least presented in the same experimental period as the US, namely during acquisition and extinction on Day 1. Similarly, the sham group showed lower startle responses and arousal ratings for CTX- and GCTX compared to CTX+, which also indicates the perception of GCTX as a safety situation. Andreatta et al. (2015b; 2017) used the same contexts, but first, had not included an extinction phase between acquisition and generalization test, and second, tested generalization directly after acquisition without overnight memory consolidation of extinction. Hence, Andreatta et al. (2015b; 2017) assessed generalization of anxiety on subjective level meaning higher ratings for GCTX compared to CTX-. However, they found generalization of safety in startle responses by similar startle responses for GCTX and CTX-. Considering the classical fear generalization study by Lissek et al. (2014), stimuli of different similarities were compared. The study revealed that in healthy participants, fear generalization stimuli, which shared 80% of similarity with CS+ were hardly generalized in startle responses, which favors very good stimulus discrimination. These findings underline the difficulty of the investigation of generalization within our anxiety

conditioning paradigm, in which the generalization context only shared 50% similarity with CTX+. Additionally, regarding the reinstatement results, the extinction memory seemed to be stronger than the anxiety memory even after reinstatement resulting in the lack of generalization. To conclude, future studies first, should include stepwise generalization contexts of 25% and 75% similarity and therefore depict a more gradually anxiety or safety generalization and second, they should test the generalization directly after acquisition without interim extinction learning.

This is the first study, which tried to disentangle tVNS effects on cue and context extinction in one single paradigm. Altogether, I found the first evidence for accelerated physiological contextual extinction by tVNS due to either an altered stimulation protocol or due to a different study paradigm than Study 2. However, generalized context-dependent cue learning hampers further conclusions about tVNS effects on fear extinction. Furthermore, the investigation of tVNS effects on generalization seems to require more specific study designs, which also control for individual differences like genetic predispositions (Mühlberger et al., 2014) to transfer advantageous learning effects to anxiety and PTSD patients in future.

6 General discussion

The overall goal of this dissertation was two-fold: first, to extend the understanding of the development of conditioned anxiety and its cortical representations during acquisition, and second to investigate the implementation of tVNS into extinction processes to enhance extinction memory and to stabilize it for reinstatement as well as generalization processes.

First, I could demonstrate the dual context representation during the acquisition of conditioned anxiety. Interestingly, healthy participants were able to discriminate between elements of a context, which were associated with an US, and elements of the same context, which have never been associated with an US. This suggests an elemental representation of the context. Besides, electro-cortical responses recorded by EEG demonstrated enhanced stimulus processing of the anxiety context compared to the safety context. This happened independently of the single elements which suggest conjunctive context representation. Therefore, I confirmed the dual representation theory by Rudy (2009; Rudy et al., 2004). Moreover, I extended research in humans (Baeuchl et al., 2015; Stout et al., 2018) by developing the flip-book paradigm which allows an integrative investigation of the two representations in one experimental design.

Second, based on extinction facilitating effects in animal research (Peña et al., 2014; Peña et al., 2013), I implemented tVNS into an anxiety conditioning protocol in humans using virtual reality (Glotzbach-Schoon et al., 2013a). Here, I found no effects of tVNS on anxiety extinction. However, I could replicate the classical results of conditioned anxiety regarding subjective ratings and physiological responses, slow extinction learning and differential subjective as well as generalized physiological reinstatement (Genheimer et

al., 2017). Considering that the underlying mechanisms of tVNS are still not fully understood, these zero findings requested further research on stimulation parameters. In Study 3, I further investigated the stimulation parameters by trying to replicate analgesic effects in humans (Busch et al., 2013). Unfortunately, neither in tonic nor in phasic pain analgesic effects could be shown which suggested non-effective stimulation parameters. Alternatively, these zero findings may be related to the insufficient knowledge about effective translation of animal cervical vagus nerve stimulation (Peña et al., 2014; Peña et al., 2013) to human cervical vagus nerve stimulation (Englot et al., 2011; George et al., 2008) to human transcutaneous vagus nerve stimulation (Burger et al., 2017; Burger et al., 2016; Genheimer et al., 2017).

In Study 4 I slightly changed stimulation parameters (i.e., prolonged stimulation) as well as the conditioning design into a cue in context conditioning paradigm (see Andreatta et al., submitted-a; Mühlberger et al., 2014). Despite successful context conditioning and a partly successful context-dependent cue learning, tVNS neither facilitated subjective extinction learning as originally expected (Burger et al., 2017; Burger et al., 2016; Peña et al., 2014; Peña et al., 2013), nor hampered return of fear or anxiety, nor reduced stimulus generalization. However, attenuated startle responses during contextual extinction in the tVNS group revealed the first physiological hint of tVNS on extinction accelerating effects in humans. Therefore, if slight changes in stimulation parameters have impacted conditioned physiological responses, further adaptions on stimulation parameters are necessary for more effective tVNS effects in humans to use the device in future anxiety therapy.

6.1 Anxiety in the context of conditioning

Considering our goal to investigate the development of fear and anxiety regarding pathological adaptation and to understand its underlying mechanisms to facilitate extinction processes, I first have to discuss the results in the light of two emotional concepts (Fanselow, 1994, 2018a; LeDoux, Phelps & Alberini, 2016).

Fanselow (2018a) subdivided the definition of emotion, more specifically fear, into four requirements: The *evolutionary or phylogenetic function* of fear prevents threat, e.g. predation, which is in line with Seligman's preparedness theory (Seligman, 1971). The presence of *antecedent conditions* like danger-predicting stimuli elicits fear, whereas *consequent conditions* are measurable changes in behavior, while the emotion is activated, including defense responses. The *circuitry* describes cerebral changes that mediate antecedent and consequent conditions like stimulus-response sequences (Fanselow, 2018a). The predator imminence model (Fanselow, 1994, 2018b) explains fear responses on approaching threats. In this light, a pre-encounter defensive state, i.e. anxiety, is elicited in an organism because of antecedent stimuli that are associated with past experiences of predation or threat. As a consequent behavior, rats, for example, changed their meal patterns; in particular, they ate fewer but bigger meals to reduce the time in the threatening area where food was provided (Fanselow, Lester & Helmstetter, 1988). This defensive mode aims to reduce the likelihood of encountering a predator. A post-encounter defensive state, i.e. fear, reduces the likelihood of being detected and attacked by a predator. Antecedent signals are crucial for the detection of a predator (i.e., the imminent threat) and consequently fear responses are elicited, e.g. freezing behavior (Fanselow, 2018a). Circa-strike defensive mode, i.e. panic, aims at surviving a predator attack. The antecedent stimulus is the imminent physical contact to the predator

accompanied by consequent behaviors like vocalization and escape attempts (Fanselow, 2018a).

Fanselow's model nicely describes the smooth transition of anxiety, fear, and panic as well as the changes in the behavioral repertoire depending on the respective emotional states. Several studies found activation of distinct brain networks (Hamm & Weike, 2005; Löw, Weymar & Hamm, 2015) in anxiety conditioning at the beginning of and during the trial (Andreatta et al., 2015a; Herrmann et al., 2016). In Fanselow's view, the onset of an anxiety context represents phasic fear; the prolonged exposure to a context however, elicits a state of sustained fear or anxiety. Interestingly, a threat of shock paradigm in humans detected fear responses like increased skin conductance responses and fear-potentiated startle responses as well as bradycardia when the shock is inevitable (Löw et al., 2015). However, when participants could actively avoid the shock, the attention was focused on the threat predicting stimulus and the avoidance behavior. As a consequence, startle responses and EEG signals, i.e. P3 amplitude, were reduced (Löw et al., 2015). One can conclude that the active intervention of threat modifies human behavior and physiological responses. Regarding Fanselow's predator imminence model (Fanselow, 1994, 2018a), Rudy's dual representation model (Rudy, 2009; Rudy et al., 2004) could be interpreted as following: Pre-encounter defensive mode could be elicited by a conjunctive representation of a context in which the whole context is associated with potential threat. In contrast, the post-encounter defensive mode might be induced by the elemental representation of a context that comprises the specific association between an element and threat. In this line, Study 1 disentangled the states of anxiety and fear by the investigation of non-threat elements and threat elements in the same anxiety context, representing pre-encounter mode and post-encounter mode, respectively. Similar to the threat of shock paradigm, participants were passively moved towards and away from

elements that they might have associated with threats during the flip-book expression without any option of avoidance. Indeed, Study 1 confirmed the dual context representation of animals in humans and suggested different modes involved in the processing of threat stimuli. However, the subjective but not physiological discrimination of threat vs. non-threat and safety elements of a context cannot be explained by Fanselow's model (Fanselow, 1994, 2018a). Study 2 and Study 4 both support the idea of a sustained state of anxiety that contains physiological as well as behavioral and subjective anxiety changes in an organism. Specifically, I found anxiety conditioning in startle response as well as in subjective ratings and successful extinction. However, I also revealed paradigm specific reinstatement effects varying between generalized and differential reinstatement for subjective and physiological responses. Notably, the distance from the threat (Fanselow, 1994, 2018a) cannot explain those subjective and physiological differences.

LeDoux, on the other hand, defines emotions as higher-order states and conscious experiences (LeDoux & Brown, 2017). Fear, in particular, can be regarded as "*conscious feeling one has when in danger*" (LeDoux & Brown, 2017, P. E2016). More precisely, LeDoux and Pine (2016) claimed the two-system view of fear and anxiety. First, the cognitive circuit mediates conscious fearful feelings that humans can subjectively report as a mental state in terms of fear or anxiety. Notably, higher-order cortical regions including the lateral and medial prefrontal cortex, parietal neocortex, and insula mediate conscious experiences, but also mediate cognitive processes such as working memory and attention (LeDoux & Brown, 2017; LeDoux & Pine, 2016). Second, the behavioral and physiological changes in body and brain describe mainly automatic defensive responses to threat, particularly named threat responses, initiated and guided by the defensive survival circuit (LeDoux & Pine, 2016). The innate fear circuit comprises lateral and

central amygdala, which mediate defensive reactions like freezing or flight, as well as lateral and basal nucleus of the amygdala and ventral striatum (nucleus accumbens, NAcc), which mediate the control of defensive actions like avoidance (LeDoux & Pine, 2016). Though the amygdala is the main component of this neural circuit, it is not alone responsible for the experience of fear (Feinstein et al., 2013). The neural anxiety circuit extends the model by including the BNST that mediates defensive behavior in uncertain dangerous situations irrespective of the direct experience of threat (LeDoux & Pine, 2016). The described two-system framework can explain why subjective reports in humans and physiological parameters dissociated. Specifically, Study 1 demonstrated elemental as well as conjunctive context representation subjectively, whereas conjunctive context representation was revealed only on electro-cortical responses. Study 2 (Genheimer et al., 2017) showed generalized reinstatement in physiological responses, but differential reinstatement in valence reports. Though both measures depict valence, the physiological reinstatement response to CTX+ and CTX- could be interpreted as a prompt body reaction to contexts which have previously been associated either directly (CTX+) or indirectly (CTX-) with the threat. The generalization of an anxiety eliciting context to similar contexts can be a strategy for the individual's integrity and survival. Cognitively, strong safety learning resulted in differential reinstatement with low responses for the safety context. On the other hand, Study 4 only indicated differential reinstatement of physiological responses which suggests high sensitivity to paradigm specific triggers for both systems.

Altogether, the results of this current dissertation support both Fanselow's predator imminence model (Fanselow, 1994, 2018b) and LeDoux's two-system framework (LeDoux & Pine, 2016) and emphasize the interplay of both theories.

Independent of Fanselow's (Fanselow, 1994, 2018b) or LeDoux's views on threat (LeDoux & Pine, 2016), conditioning experiments are frequently used to investigate fear and anxiety or defensive responses (see Lonsdorf et al., 2017). Nevertheless, conditioning paradigms are also criticized as experimental models for pathological fear or anxiety responses (Beckers, Krypotos, Boddez, Effting & Kindt, 2013). During conditioning, attentive participants will mostly learn the association of a cue predicting an aversive event and subsequently they will show fear or anxiety responses. This can be regarded as normal adaptive behavior advantageous for the organisms' health. In everyday life, people may be confronted with aversive, or even traumatic events but do not necessarily develop an anxiety disorder (Engelhard, de Jong, van den Hout & van Overveld, 2009). However, when a person develops pathological fear or anxiety, he or she might show excessive avoidance or exceedingly high levels of fear or anxiety responses (American Psychiatric Association, 2013) rather than small responses to slightly threatening events or situations (Beckers et al., 2013). Despite such considerations, conditioning paradigms comprise many advantages for the investigation and understanding of biological mechanisms, risk factors and individual differences in fear learning (Lonsdorf & Merz, 2017). Therefore, fear and anxiety conditioning are frequently chosen as research models for investigating acquisition, extinction, and return of fear (and anxiety) phenomena (see Lonsdorf et al., 2017).

Classical conditioning has many advantages including the variety of learning protocols, the translational value of the research and the investigation on the etiology as well as maintenance of the pathogenesis of anxiety. First, researchers can select an established animal or human experimental protocol for their research questions. In this way, the flip-book paradigm of Study 1 was developed by benefitting from context conditioning in virtual reality (Glotzbach-Schoon et al., 2013a), context representation studies (Baeuchl

et al., 2015; Stout et al., 2018) and by methodological investigations of multiCS conditioning EEG studies (Bröckelmann et al., 2011; Steinberg et al., 2013). Second, researchers can investigate normal, abnormal and pathological behavior by applying animal models, by examining patients directly or by comparing experimental manipulations in healthy human individuals. I used context conditioning in Study 2 (Genheimer et al., 2017) since the model fit best to the requirements to elicit the sustained state of anxiety in participants and to investigate underlying mechanisms of extinction learning under controlled conditions (Bouton, 1994; Bouton, 2002; Genheimer et al., 2017; Glotzbach-Schoon et al., 2013a; Grillon et al., 2008). In this line, Glotzbach-Schoon et al. (2015), as well as Study 2 (Genheimer et al., 2017), pointed out the influence of state anxiety on reinstatement. Both revealed generalized reinstatement in startle response for low anxious individuals, but differential reinstatement in high anxious individuals. One explanation might be the mood congruency effect (Glotzbach-Schoon et al., 2015; Lewis & Critchley, 2003). This describes that a specific memory is more likely to be retrieved when a person is in the same mood as he or she was when the memory was encoded. Hence, during reinstatement, high anxious participants might recall the anxiety memory, which has previously been encoded during acquisition, whereas participants with low state anxiety might recall the extinction memory. Third, experimental conditioning protocols can model the pathogenesis of anxiety and PTSD (Jovanovic et al., 2013; Mineka & Zinbarg, 2006). Thus, Lissek et al. (2005) showed in their meta-analysis stronger fear responses to the CS in anxiety patients in single cue conditioning paradigms and impaired discrimination learning in differential conditioning paradigms. In particular, they exhibit increased fear responses also to CS- and impaired extinction learning compared to healthy controls (Blechert, Michael, Vriends, Margraf & Wilhelm, 2007; Duits et al., 2015; Lissek et al., 2005). During safety learning, the amygdala is inhibited by the prefrontal

cortex like during extinction learning (Lindner et al., 2015; Myers & Davis, 2007). The inability to inhibit fear responses during safety cues can be tested by a summation test protocol (Jovanovic et al., 2013). In this paradigm, stimulus A (CS+) and stimulus B (CS-) both are presented simultaneously with a third stimulus X. During acquisition, AX is paired with an US, but not BX. Attenuated startle responses are observed during a test phase, in which A and B are presented as a compound, which is indicative of B as a simultaneously conditioned inhibitor (Jovanovic et al., 2013; Myers & Davis, 2004; Wendt, Neubert, Koenig, Thayer & Hamm, 2015). In this way, impairment of safety learning has been investigated in anxiety and PTSD patients. Fourth, stimulus generalization and categorization mechanisms are further concepts that can be investigated by experimental conditioning paradigms in healthy individuals or anxiety patients. One prominent paradigm of fear generalization protocols uses two neutral female facial expressions as CS+ and CS- and its morphs for the presentation of generalization stimuli (see Britton et al., 2011; Schiele et al., 2016). Commonly, anxiety patients compared to healthy controls generalize their fear of similar objects (Lissek et al., 2014). We were interested whether conditioned fear responses would be generalized in a new ambiguous context. In Study 4 I, therefore, used two virtual contexts (CTX+ and CTX-) and a generalization context which was the equal mix of the other two. In this study, I did not observe the generalization of conditioned fear. However, this zero finding may be related to the fact that the participants were healthy and may have presented quick extinction learning (in fact, the CTX+–US contingency was greatly reduced during generalization phases) which in turn prevented fear generalization.

Vervliet and Raes (2013) discussed the external validity and quality of such experimental protocols. The predictive validity of an experimental protocol including fear conditioning describes the prediction of treatment outcome in patients based on the

experimental performance in the laboratory and vice versa (see also Boddez et al., 2013). Construct validity, on the other hand, refers to the underlying theory and the question of whether the pathological mechanisms are appropriately described in the model (Beckers et al., 2013). Importantly, the diagnostic validity of the model taps into the differences between at-risk individuals, patients, and healthy humans and provides insights into the etiology of the disorder (Boddez et al., 2013; Vervliet & Raes, 2013). As nicely stated by Boddez et al. (2013, P. 202) '*face validity of a test or model refers to the surface similarity between the test and the condition that the test aims to model*'. One example of face validity is US expectancy ratings. Thus, participants in the laboratory are asked for their expectancy of the aversive stimulus at certain times during the conditioning experiment (Boddez et al., 2013). Similarly, anxiety patients can communicate their anticipation of an aversive event (Boddez et al., 2013). Examples include dog phobia patients expecting to be bitten when confronted with a dog (Thorpe & Salkovskis, 1995), panic patients expecting a panic attack due to distinct interoceptive or exteroceptive stimuli that were associated with a previous attack (Bouton et al., 2001) or generalized anxiety patients expecting threat in a certain context (Vansteenwegen et al., 2008). Such face validity was of special interest in Studies 2, 3 and 4 because transcutaneous vagus nerve stimulation was investigated as a possible add-on tool for exposure therapy. If face validity is assured, results are transferable from healthy individuals to patients.

To this end, predictive, construct, diagnostic and face validity are essential for the selection of the appropriate experimental paradigm to investigate an individual research question. Classical conditioning, which is a basic associative learning procedure, provides not only a good starting point to investigate fear and anxiety responses but it is also a powerful experimental model for extinction, generalization, and return of fear. In this line, existing conditioning paradigms require some extensions for modeling the appropriate

circumstances in the laboratory. For instance, the particular investigation of the elemental and conjunctive representation of a context in humans (Baeuchl et al., 2015; Stout et al., 2018) required changes and adaptions in the paradigm. Furthermore, the selection of appropriate measures can be pivotal for the results and adequate conclusions for healthy participants as well as for the translation of the results to further studies with e.g. patients (see Study 1 and Study 4).

Of special interest in this current work was the acquisition, extinction, generalization, and return of anxiety in context conditioning experiments. For this purpose, some conditioning studies used the technology of virtual reality for the high control of experimental conditions and the ecological validity of the setup (Glotzbach-Schoon et al., 2013a). First, the development of a merged cue and context conditioning paradigm was possible in Study 1 because of the VR technology. By presenting and disentangling cues in contextual manner elemental as well as conjunctive context representation was achieved. The investigation of such underlying mechanisms in a realistic way is otherwise hardly possible. Second, for the realization of anxiety conditioning paradigms and in particular for the elicitation of sustained anxiety in the laboratory, the presentation of whole contexts promises higher ecological validity (Bohil et al., 2011) compared to any artificially created context like the presentation of background colors (Pohlack et al., 2012). Third, the investigation of generalization is another main topic in understanding fear and anxiety in humans (see Study 4). For fear generalization, fear conditioning paradigms are well established using geometric shapes or faces (Dymond et al., 2015; Lissek et al., 2008; Schiele et al., 2016). Anxiety generalization, however, is hard to realize in laboratory settings because a context consisting of two other contexts contains much more information than a single picture or color of a light can provide. Nevertheless, research in generalization is highly relevant as many mental health disorders like

generalized anxiety disorder, obsessive-compulsive disorder or specific phobia have highly recurrent symptoms (Dymond et al., 2015). In this case, VR increases the discrimination capacity of different contexts and even enables researchers to create a gradient of similarities of the generalization context to anxiety and safety context (Andreatta et al., submitted-b). In Study 4, I also took particular advantage of VR. Instead of creating a novel context in the test phase (Mühlberger et al., 2014) I used equal parts of CTX+ and CTX- to investigate the competition of the anxiety and the extinction memory trace in a return of fear paradigm.

Context per se turned out to contain an outstanding information value, not only in the described anxiety acquisition but also in extinction processes and return of anxiety. Thereby, I mainly focused on spatial and partly on temporal contexts but neglected any interoceptive, cognitive, social and cultural context (Maren et al., 2013). However, one can assume that all of these contexts influence the anxiety conditioning model. The contingency awareness, in contrast, did not seem to influence our results on context conditioning, though some participants did not learn which of the two offices or lights in an office respectively, were associated with the aversive US.

Altogether, I modeled anxiety behavior in healthy humans by applying conditioning paradigms in VR and investigated contextual representation during acquisition (Study 1) as well as extinction and return of fear and anxiety phenomena with additional generalization (Study 2 and 4). For this purpose, anxiety conditioning allowed us to expand existing knowledge, especially in human anxiety.

6.2 Vagus nerve stimulation

Vagus nerve stimulation has been used in a variety of experiments on fear and anxiety in animals (Alvarez-Dieppa et al., 2016; Childs et al., 2015; Noble et al., 2017; Peña et al.,

2014; Peña et al., 2013) as well as in humans (Burger et al., 2017; Burger et al., 2016; Genheimer et al., 2017; George et al., 2008). Importantly, side effects reported by the participants were extremely low in current experiments: tingling, itching sensations and slight, temporary headache were the most common symptoms participants reported in Studies 2, 3 and 4. No further negative long-term effects emerged. This is in line with other studies that reported minimal side effects (Burger et al., 2016; Busch et al., 2013; Fischer et al., 2018; Ventura-Bort et al., 2018). Therefore, tVNS is a well tolerable stimulation method that might help patients of several diseases in the future.

For a successful application in clinical settings, some issues emerged from the studies of this thesis. First, the optimal stimulation intensity is still under debate. A study in rats by Clark et al. (1995) compared the effects of VNS of different stimulation intensities on memory. All rats underwent surgery to implant a VNS device. After recovery, they were trained in one-trial learning to receive a foot-shock in one part of a runway. Thereafter, rats received either 0.2 mA, 0.4 mA, 0.8 mA or the control group 0 mA VNS. Interestingly, one day later, rats with 0.4 mA stimulation showed more avoidance of the threatening part of the runway compared to all other groups, which was interpreted as better memory retention. Therefore, the authors suggested an inverted U-shaped curve as the best outcome for VNS effects (Clark et al., 1995). In line with those results is a study in epilepsy patients who wore an implanted vagus nerve stimulator (Clark, Naritoku, Smith, Browning & Jensen, 1999). Patients, whose vagus nerve was stimulated after a memory task with a medium intensity of 0.5 mA, showed better retention compared to patients, who were stimulated with high or low intensities, i.e. 0 mA or 0.75-1.5 mA (Clark et al., 1999). In this line, Helmstaedter, Hoppe, and Elger (2001) found the best performance in a recognition task in epilepsy patients that were stimulated with medium intensity. Higher intensities of 0.75-1.5 mA turned out to impair the performance in the memory

recognition task. However, sample sizes in these clinical studies were very small with 5 (Clark et al., 1999) or 11 (Helmstaedter et al., 2001) patients per stimulation group and therefore, these findings should be treated cautiously. However, a systematic investigation of the stimulation intensities for tVNS is still missing. The company cerbomed GmbH (Erlangen), which developed the transcutaneous vagus nerve stimulation device, suggests the best stimulation for positive effects on epilepsy patients when patients feel a tingling sensation but no pain. However, those specifications of the application of stimulation parameters are not sufficiently precise. For this reason, some studies (Burger et al., 2017; Burger et al., 2016) stuck by a stimulation intensity of 0.5 mA for all participants. However, individual differences like skin conductance of the ear or pain sensitivity should be considered to establish the optimal stimulation. I tried to overcome this issue by determining each participant's individual stimulation intensity. Surely, this is one strength of the current work and a first step towards the consideration of individual differences. Although the procedure has been successfully applied in several other studies (Fischer et al., 2018; Ventura-Bort et al., 2018) it was not effective in my works. It is possible that my stimulation intensities of on average 1.1 – 1.3 mA in Studies 2, 3 and 4 were too high. As a consequence, I speculate that this might have elicited a high release of NE into the synaptic cleft which would boost excitatory signal transduction. Therefore, the systematic investigation of the comparability of cervical and transcutaneous VNS intensities and the optimal stimulation intensity to induce neural plasticity has to be investigated in the future.

The second issue is the manipulation check for tVNS. Some procedures have already been suggested and discussed including vagus sensory evoked EEG potentials (Fallgatter, 2003) or pupil dilation (Jodoin et al., 2015). Clancy et al. (2014) for example assessed heart rate variability (HRV) as a measure of parasympathetic activity and found

increasing HRV mediated by tVNS. A systematic investigation by De Couck et al. (2017) considering long and short tVNS periods as well as left and right concha stimulation found changes in HRV after a short stimulation period of 10 min only, when the right concha was stimulated. Interestingly, prolonged stimulation (1 h) of the right cymba concha increased HRV only in females, but not in males (De Couck et al., 2017). This confirms anatomical findings of higher innervation rates of the right vagus and the heart compared to the left vagus and the heart (Schachter & Saper, 1998). Since experiments specifically aim to reduce cardiac effects and therefore only stimulate the left branch of the vagus (George, 2000), HRV seems to be a less-than-ideal manipulation check. Alternatively, pain perception may be a better manipulation check (Busch et al., 2013). This was the purpose of Study 3, but I found no analgesic effects on tonic pain as found by Busch et al. (2013). One trivial explanation may be that our stimulation in Studies 2 and 3 did not work and we, therefore, did neither find accelerated extinction nor analgesic effects. However, this seems unlikely as we used the same stimulation device and similar parameters as Busch et al. (2013) did. Another more plausible explanation is the slight changes in our experimental setup compared to Busch et al. (2013). While Busch et al. (2013) used a within-subject design and a test battery of quantitative sensory testing (QST), I used a between-subjects design and tested only pressure pain as a comparable variable. Although Study 3 does not allow conclusions on the optimal stimulation parameters, the application of the tVNS in clinical settings is still striking. More robust effects are still desirable. This includes general requirements for any manipulation check comprising reliability as well as the easy and fast add-on application to every study design. For this purpose, salivary alpha-amylase (sAA) seems to be the most reliable manipulation check at the moment. Chatterton, Vogelsong, Lu, Ellman, and Hudgens (1996) discovered sAA as a biomarker for catecholamines, i.e. NE levels, in plasma and reported a more direct

measure of catecholamine activity than changes in heart rate. Specifically, the higher sAA concentrations are the more NE was found in the plasma (Chatterton et al., 1996). The increase of sAA concentrations is caused by physical stress in interval training in sports (Walsh et al., 1999) but also by psychosocial stress like an exam situation (Chatterton et al., 1996). Nater and Rohleder (2009) reviewed the literature confirming sAA as a biomarker for sympathetic activity. Though Carpenter et al. (2004) could not find differences in sAA levels between a vagus nerve and a sham stimulated group, Fischer et al. (2018) and Ventura-Bort et al. (2018) recently found evidence for higher sAA concentrations when the vagus nerve was stimulated as compared to sham stimulation. As described above, tVNS activates the LC in the brain stem which releases the neurotransmitter NE into other brain areas (Ben-Menachem et al., 1995; Raedt et al., 2011; Roosevelt et al., 2006; Van Bockstaele et al., 1999). The collection of salivary samples and the measurement of sAA concentration takes advantage of this mechanism. Additionally, the procedure of saliva sample collection is very simple and quick and can easily be applied in future studies. Although Ventura-Bort et al. (2018) also found them, sAA effects are still very weak and these findings need to be replicated in additional, larger samples. However, their results support the idea that sAA concentration might be the state-of-the-art manipulation check for tVNS.

A third very general issue of research on tVNS effects are discussions about gender differences. Burger et al. (2018) and Ventura-Bort et al. (2018) raised this discussion on the morphology of the vagus nerve in animals and humans. At first glance, it seems irrelevant to control tVNS research for gender effects as it was the case in many studies including Study 3, studies on cognitive processes (Beste et al., 2016; Ventura-Bort et al., 2018), action cascade processing (Steenbergen, 2015), flow experiences (Colzato, Wolters & Peifer, 2018) and associative learning (Burger et al., 2017; Burger et al., 2016).

However, in all of these studies, the number of female participants was higher than the number of male participants. Indeed, gender differences were found in the vagus nerve anatomy (Moriyama, Hayashi, Inoue, Itoh & Otsuka, 2016) and the LC-NE system (reviewed in Bangasser, Wiersielis & Khantsis, 2016). For instance, the LC was found to be larger and containing a bigger dendritic arbor in female compared to male rats (Bangasser et al., 2016; Bangasser, Zhang, Garachh, Hanhauser & Valentino, 2011; Pinos et al., 2001). This is related to more pronounced neurogenesis in LC during puberty in females, but not in males (Pinos et al., 2001). Two studies (Busch, Bohl & Ohm, 1997; Ohm, Busch & Bohl, 1997) counted the LC neurons in women and men resulting in a higher number of neurons in females' LC (Bangasser et al., 2016). More complex dendritic trees in the LC could result in higher NE concentrations and therefore higher arousal in females, especially in emotional events (Bangasser et al., 2016). Moreover, rat studies showed that the estrous cycle influences NE concentrations (Selmanoff, Pramik-Holdaway & Weiner, 1976) and estrogen increases NE synthesis and decreases NE degradation (Vathy & Etgen, 1988). This potential imbalance of NE might also be one reason for the higher numbers of stress-related disorders in females (Bangasser et al., 2016). Lastly, weakly stressful events were found to activate the LC in females through the neuropeptide corticotropin-releasing factor (CRF), but not in males suggesting higher CRF sensitivity (Curtis, Bethea & Valentino, 2005; Hauger et al., 2012). In sum, several animal studies investigating morphology and physiology of the vagus nerve and its LC projections found evidence for gender differences. However, human studies are still sparse. Previous studies on associative memory, which included a similar number of male and female participants, did not report any gender differences in the results (Jacobs, Riphagen, Razat, Wiese & Sack, 2015). Considering current results, samples including the same number of male and female participants (see Study 2 and 4) did not reveal any differences in extinction

memory or retention due to gender. In the light of anatomical studies in animals, human research on vagus nerve stimulation should consider gender as a potential mediating factor for the stimulation effects.

One further aspect of using tVNS, which should be taken into account, is the arousal. As described above, emotional arousal induces higher attention and physiological changes in the brain thereby modulating memory processes (McIntyre et al., 2012). TVNS increases NE release in the brain which in turn is related to high emotional arousal (see Peña et al., 2013). Inducing emotional arousal through the experimental manipulation might overlap with the tVNS effects (Burger et al., 2018). Possibly, fear conditioning studies may induce an additional arousal level due to the delivery of the US. Conceivably, studies using low arousing cues (e.g. geometrical shape) and predictable learning paradigms (i.e., classical fear conditioning) found facilitated extinction in tVNS stimulated participants (Burger et al., 2016). In contrast, studies using high arousing stimuli such as pictures of spiders found facilitated fear acquisition (Ho & Lipp, 2014), but hampered effects of tVNS resulting in similar extinction patterns for verum and sham stimulated participants (Burger et al., 2018). In line with this argumentation, the use of virtual reality technology might also have been highly arousing and consequently disadvantageous for the tVNS effects. A replication of a VR study in less arousing contexts by using e.g. empty rooms, distinguishable through different colors of the lights, as implemented in a study by Shiban et al. (2013) would be interesting.

Notably, genetic variation could not only affect fear and anxiety conditioning (Glotzbach-Schoon et al., 2013b; Heitland et al., 2013; Lonsdorf & Baas, 2017; Mühlberger et al., 2014), but also the LC-NE system (Mueller & Cahill, 2010). More specifically, the norepinephrine transporter (NET) regulates the NE homeostasis by NE reuptake from extracellular levels into the pre-synapse (Iversen, 1963; Xu et al., 2000). Regarding

epigenetics, hypermethylation of the promoter region of the NET gene decreases the number of norepinephrine transporters, which hampers NE reuptake (Esler et al., 2004). Interestingly, increased DNA methylation of the NET gene was found in panic disorder patients compared to healthy controls resulting in augmented neural stimulation in patients (Esler et al., 2006). Possibly, individual genetic methylation patterns might also result in different impacts of the vagus nerve stimulation.

In sum, the approaches of tVNS usage in various fields are very promising but still are in the early stages of development. More basic as well as applied research in animals as well as in healthy humans and patients is necessary to better understand the underlying mechanisms and to exhaust the diversity of implementations.

6.3 Vagus nerve stimulation and Extinction

A specific goal of the current work was the investigation of vagus nerve stimulation on extinction learning and its effects on the return of conditioned anxiety and fear generalization. One mechanism that could affect extinction is described in the polyvagal theory (Porges, 2009). Specifically, stimulation of the vagus nerve, which is part of the parasympathetic nervous system, inhibits the activity of the sympathetic nervous system which is referred to as vagal brake (O'Keane, Dinan, Scott & Corcoran, 2005; Porges, 2009). Consequently, the heart rate is slowed down (Higgins, Vatner & Braunwald, 1973) and VNS has anxiolytic (Peña et al., 2013) and mood-improving effects (George et al., 2008). In contrast, Peña et al. (2014; 2013) explained their findings of facilitated fear extinction by increased emotional arousal which enhanced neural plasticity and memory (Bradley, Greenwald, Petry & Lang, 1992; Cahill & McGaugh, 1998). Fanselow (2013) and Peña et al. (2013) described the mechanism of enhanced memory consolidation at least partially mediated by a neural pathway from the autonomous nervous system to the

amygdala. More specifically, during emotionally arousing or stressful conditions, epinephrine is released from the adrenal gland. Epinephrine can bind to the β-adrenoceptors at the vagus nerve. Vagal afferents in turn project to the brain stem nuclei and induce the release of neurotransmitters including norepinephrine to NTS, LC, amygdala, PFC and hippocampus (Dorr & Debonnel, 2006; Hassert et al., 2004; Miyashita & Williams, 2004; Miyashita & Williams, 2006; Roosevelt et al., 2006). In this line, increased NE levels in the amygdala were found after exposing a rat to an aversive stimulus, after the administration of epinephrine and after stimulation of the vagus nerve (Hassert et al., 2004; McIntyre, 2002; Williams, Men, Clayton & Gold, 1998). MRI studies in humans support these mechanisms observed in rodents and provide evidence for brainstem activity including NTS, LC, parabrachial area, dorsal raphe, periaqueductal gray, thalamus, amygdala, insula, nucleus accumbens, and BNST subsequently to tVNS (Dietrich et al., 2008; Frangos et al., 2015). Therefore, stimulation of the vagus nerve skips the emotional arousal or stress and directly enhances memory consolidation by NE release in the brain.

Studies in rats have already revealed a reliable extinction facilitation and stabilization effect of cervical VNS (Alvarez-Dieppa et al., 2016; Childs et al., 2017; Noble et al., 2017; Peña et al., 2014; Peña et al., 2013). After auditory fear conditioning, VNS attenuated freezing responses to the threatening cue even two weeks after the last treatment (Peña et al., 2013) and enhanced neural plasticity of the infralimbic-amygdala pathway thereby inhibiting the conditioned fear response (Peña et al., 2014). The underlying mechanisms involve increased protein expression of GluN2B and enhanced phosphorylation of calcium/calmodulin-dependent protein kinase (CaMKII) in BLA, both associated with enhanced neural plasticity (Alvarez-Dieppa et al., 2016), and reversed impairment of extinction as well as decreased reinstatement and attenuated symptoms of hyperarousal,

social avoidance and anxiety in a rat PTSD model (Noble et al., 2017). In sum, animal studies could successfully demonstrate the impact of cervical VNS on extinction learning.

Several studies have already tried to translate the results in animals to humans using tVNS (Burger et al., 2017; Burger et al., 2016; Genheimer et al., 2017). Cue conditioning was applied as a model to enlighten fear expression during acquisition and extinction (Burger et al., 2018; Burger et al., 2017; Burger et al., 2016). The most interesting effect was revealed in the online contingency ratings, when the CS was low but not when the CS was high arousing (Burger et al., 2018). The former effect refers to the tVNS accelerated extinction as indicated by lower contingency ratings in the tVNS compared to a sham stimulated group (Burger et al., 2017; Burger et al., 2016). The latter effect refers to the missing replication of this quicker verbal extinction, when the CSs were highly arousing, i.e. when spider pictures were used (Burger et al., 2018). In all studies (Burger et al., 2018; Burger et al., 2017; Burger et al., 2016) no physiological effects were indicated. Such dissociation between verbal and physiological responses supports the two-system model which suggests two memory systems: the subjective system, which relies on the hippocampus, and the physiological system, which relies on the amygdala (LeDoux & Pine, 2016; Phelps, 2004). Burger et al. (2017) suggested greater changes in the hippocampus than in the amygdala. Animal studies indeed revealed hippocampus activation induced by VNS (Biggio et al., 2009; Dorr & Debonnel, 2006) which supports Burger et al.'s (2017) hypothesis of enhanced declarative memory.

In comparison to the extinction of conditioned fear, the effects of VNS on the extinction of conditioned anxiety are even less investigated. The only results of context conditioning and VNS in rats were provided by Peña et al. (2014), who called their experiment an auditory fear conditioning paradigm. In this experiment, a short electric footshock was delivered randomly when a tone was presented for 30 s and they additionally investigated

freezing behavior during the inter-tone-interval for the assessment of contextual anxiety. Attenuated freezing in VNS compared to sham stimulated rats during extinction was interpreted as accelerated anxiety extinction (Peña et al., 2014). As the hippocampus entails contextual information, activation of the hippocampus by VNS could have facilitated anxiety extinction (Biggio et al., 2009; Dorr & Debonnel, 2006). Study 2 of the current dissertation (Genheimer et al., 2017) was the first to investigate tVNS effects on anxiety extinction and the return of anxiety in humans. Besides successful acquisition, extinction and reinstatement of conditioned anxiety, no effects of tVNS were found. Interestingly, some human MRI studies showed no hippocampus activation while some revealed even a deactivation (Dietrich et al., 2008; Frangos et al., 2015; Kraus, 2007). Interestingly, Kraus (2007) found amygdala deactivation induced by tVNS in humans. Considering that both the amygdala and the hippocampus are needed for contextual representation (Rudy, 2009) and that the hippocampus is deactivated by tVNS (Frangos et al., 2015), anxiety extinction in the sham stimulated group should be facilitated. However, this was not the case (Genheimer et al., 2017). Supportively, the context-dependent extinction of conditioned fear in Study 4 was also not affected by tVNS. Until now, the results of activated brain patterns due to tVNS in humans are still inconsistent and have to be better investigated in the future. Then, cue and context conditioning studies in healthy humans as well as in anxiety patients are warranted, as vagus nerve stimulation could indeed reveal a successful new treatment method for anxiety patients.

6.4 Implications of tVNS for exposure-based therapy

Anxiety patients, clinicians and even the public health system call for the most effective therapy with only few individual-focused sessions, best and sustained treatment success, including low costs, optimal outcome and low risk of relapse. Multiple approaches are

already applied in exposure therapy (for a review see Craske et al., 2018). Subsequent aims of my research were first to better understand the mechanisms underlying fear or anxiety learnings, second to optimize therapy outcome by consideration of mechanistic extinction processes and third to improve consolidation of therapeutic content to avoid relapses after therapy. Fanselow (2018b) suggested that during a threatening situation the organism's limited behavioral repertoire to only the phylogenetically most relevant behaviors for survival hampers extinction learning because of the loss of learning flexibility. In this line, PTSD patients showed impaired extinction recall (Milad et al., 2008; Milad et al., 2009) as well as reduced vmPFC and increased amygdala activity (Hayes, VanElzakker & Shin, 2012; Shin et al., 1999; Stevens et al., 2013). Supportively, Rougemont-Bücking et al. (2011) found reduced vmPFC activity to the extinction context during extinction recall in PTSD patients which might by an index for impaired processing of extinguished cues and extinguished contexts in PTSD patients.

One treatment option for patients is pharmacotherapy. However, a working group in the USA called out a crisis in pharmacotherapy treatment of PTSD (Krystal et al., 2017). Reasons are complex: first, in the USA, the only two drugs (sertraline and paroxetine) approved by the Federal Drug Association (FDA) for PTSD treatment simply hamper symptoms and have strong side effects. Second, several medications are prescribed to these patients to reduce single symptoms like anxiety, depression, chronic pain, sexual dysfunction, and sleeping difficulties. However, the interaction of all these drugs is unknown. Third, Krystal and colleagues (2017) report a lack of the development of new medication since 2001 (see McIntyre, 2018).

Currently, exposure-based therapies are a well-established treatment option for PTSD (Craske et al., 2008; Foa et al., 1999). Foa et al. (1999) compared therapeutic approaches in female assault victims and found improvement in all kinds of therapy as compared to a

waiting list control group with the best outcomes of prolonged exposure therapy. Virtual exposure therapy has comparable effects as in vivo therapy (DiMauro, 2014; Gonçalves, Pedrozo, Coutinho, Figueira & Ventura, 2012; Gromer, Reinke, Christner & Pauli, 2019). The majority (80%) of Iraq/Afghanistan war veterans suffering from PTSD treated with a virtual reality exposure therapy (VRET) reported improved PTSD symptoms resulting in a better daily life quality (Rizzo et al., 2010).

Craske and colleagues suggest various approaches to maximize the outcome of such exposure-based therapy like deepened extinction, retrieval cues or multiple contexts exposure (Craske et al., 2008; Craske et al., 2014). However, many investigations and recent reviews emphasize the high numbers of non-responders, drop-outs and the problem of standardized protocols for individual patients who might need very specific exposure treatment (Kar, 2011; Lonsdorf & Merz, 2017; Rizzo et al., 2010; Schottenbauer, Glass, Arnkoff, Tendick & Gray, 2008; Steenkamp, Litz, Hoge & Marmar, 2015). Furthermore, relapse of successfully treated patients is another relevant challenge for researchers and psychotherapists (Boschen, Neumann & Waters, 2009). Therefore, to prevent relapses or to develop a more individualized therapeutic approach, the investigation of underlying acquisition and extinction processes of conditioned fear as well as anxiety is inevitable and urgent.

One promising approach for improving exposure therapy was suggested by Walker et al. (2002) and Davis et al. (2006). They administered cognition-enhancing D-cycloserine to rats before extinction which enhanced extinction retention in terms of less freezing responses. In a second step, the same drug was administered to anxiety patients who showed less fear of heights after exposure therapy. However, the administration of cognitive enhancers in studies with PTSD patients revealed mixed results (Litz et al., 2012; Rothbaum et al., 2014). McIntyre (2018) discussed the effects of drug

administration (D-cycloserine) before exposure training when anxiety was elicited by the expectation of being exposed to the situation in which a traumatic event happened (McIntyre, 2018). Moreover, anxiolytic drugs might, on the one hand, reduce anxiety before and within an exposure therapy session. On the other hand, D-cycloserine did not affect extinction learning (Rothbaum et al., 2014). Possibly, elicitation of anxiety responses seems fundamental during the exposure so that patients can learn to handle, to accept and to decrease their anxious reaction. It is also conceivable that extinction memory trace, created during exposure therapy, is enhanced by the elicited stress-related anxiety responses, which have been found to facilitate memory consolidation (LaLumiere, McGaugh & McIntyre, 2017; Tuerk et al., 2018). According to McIntyre (2018), optimal treatment taps *'into the mechanisms that enhance the consolidation of traumatic memories in order to promote extinction memories that are just as strong, all the while bypassing or avoiding the aversive stress response*' (McIntyre, 2018, P. 96). Therefore, a more precise knowledge of the underlying mechanisms as well as an appropriate and effective development of a tVNS is a promising tool that gives hope for improvement in the treatment of anxiety and PTSD patients. Especially PTSD patients might profit from tVNS because they show lower general states of arousal compared to anxiety disorder patients (American Psychiatric Association, 2013). Although the modulation of VNS on cue conditioning has been extensively investigated in animals, investigations on context conditioning are still sparse (Alvarez-Dieppa et al., 2016; Noble et al., 2017; Peña et al., 2014; Peña et al., 2013). Interestingly, patients suffering from refractory epilepsy and wearing an implanted VNS reported increased quality of life (Dodrill & Morris, 2001). Supportively, George et al. (2008) implantation of VNS in anxiety patients seems also to alleviate the symptomatology (George et al., 2008). Some weak, but promising effects of tVNS on fear and anxiety extinction in healthy humans have been found by myself (Study

2 and Study 4; Genheimer et al., 2017) and by others (Burger et al., 2017; Burger et al., 2016; Genheimer et al., 2017). However, further and more reliable results are required for its clinical application.

6.5 VNS, Cognitive functions and other applications

Considering fear extinction studies, Burger and colleagues (2017; 2016) found facilitated extinction by tVNS only on the level of declarative memory. Besides the pivotal NE-LC system, GABA and acetylcholine (ACh) are also two highly important neurotransmitters for cognitive functioning (Van Leusden, Sellaro & Colzato, 2015). On the one hand, the GABAergic system is suggested to modulate memory consolidation (Ghacibeh, Shenker, Shenal, Uthman & Heilman, 2006), to affect bistable perception (van Loon et al., 2013) and to ameliorate performance in action cascading (Yildiz et al., 2014). On the other hand, the release of ACh mediated by VNS controls movements and actions (Watanabe, Shimizu & Matsumoto, 1990), stimulus encoding and action coordination (Bartus, Dean, Beer & Lippa, 1982). Thus, altered memory performance and cognitive functioning by tVNS could improve treatment for several diseases. Interestingly, VNS might affect rehabilitation of stroke (Khodaparast et al., 2014; Khodaparast et al., 2013), tinnitus (Kilgard, 2012; Tyler et al., 2017), major depression (Fang et al., 2017; Sackeim et al., 2001) and obesity patients (Berthoud, 2008; Bodenlos et al., 2007).

Ventura-Bort et al. (2018) investigated the attentional processes by measuring the electro-cortical activity of P3 amplitudes in an oddball-task. Specifically, standard pictures of ovals were used for the irrelevant and frequently presented condition. Besides, for the easy condition, ovals depicting schematic rotated-heads shown from above were presented with a nose pointing upwards and one ear. Participants had to indicate whether the ear was on the left or right side by button press. In the difficult condition, the nose was

shown downwards. Participants had to indicate the side of the ear from the perspective of the head. Since the P3b component reflects evaluation processes of a stimulus during decision making and memory processes (Donchin & Coles, 1988; Polich, 2007; Ventura-Bort et al., 2018) and is likely to be modulated by phasic responses of the LC-NE system (Nieuwenhuis, Aston-Jones & Cohen, 2005; Polich, 2007), it was expected that tVNS modulates P3b during the oddball-task. Indeed, results revealed increased P3b amplitudes elicited by easy targets, i.e. heads with the nose up, in the tVNS compared to sham stimulated participants, however not in the difficult nor in the novel condition (Ventura-Bort et al., 2018). Interestingly, tVNS did not affect behavioral responses such as reaction times (Ventura-Bort et al., 2018). The authors explain the results of comparable P3b amplitudes between easy and difficult tasks as well as higher reaction times and lower performance accuracy in the difficult condition due to the mental rotation process which requires more spatial working memory resources. As a result, the enhanced neuronal processing generated by the mental rotation in the difficult task might overlap with the modulation of the ERPs by vagus stimulation and enlarged arousal levels (Polich & Kok, 1995), resulting in a ceiling effect of tVNS for the difficult target condition (Ventura-Bort et al., 2018). An alternative explanation might be increased emotional arousal in the difficult task which could also have overlapped with the effects induced by tVNS as suggested previously (Burger et al., 2018; Burger et al., 2017). Another study by Jacobs et al. (2015) considered associative memory in healthy elderly participants of about 60 years. Interestingly, tVNS stimulated participants showed a better performance in a memory task than sham stimulated participants, meaning they remembered the names of previously seen faces better. As a translation of these results to the clinical setting, tVNS might also be a potentially promising treatment for Alzheimer's disease (AD).

Following this idea, AD development seems to increase with age because of the changes in the immune system which increase the risk for inflammatory responses such as an elevation in cytokines in the body (Daulatzai, 2012). The NTS is very vulnerable for inflammation, which could result in cell death of LC neurons, which in turn reduces NE release and anti-inflammatory protection possibly leading to neurodegeneration processes (Daulatzai, 2012). In line with this theory, two studies have shown that long-term implanted VNS counteracts NE reduction and inflammation (Merrill et al., 2006; Sjögren et al., 2002). A second theory about VNS effects on AD proposes that VNS and increased NE levels promote the synthesis of neurotrophic factors, which in turn increases cell proliferation (Follesa et al., 2007; Patel, Chen & Russo-Neustadt, 2010; Revesz, Tjernstrom, Ben-Menachem & Thorlin, 2008). However, Vonck et al. (2014) emphasized the lack of research on cell proliferation as well as on survival rate.

Altogether, it seems that vagus nerve stimulation not only improves safety and extinction learning, but it is also beneficial for the treatment of a variety of diseases.

6.6 Limitations and outlook

As mentioned before, the most important limitation of current studies on tVNS is the lack of a reliable manipulation check. As Study 3 did not replicate the analgesic effects of tVNS (Busch et al., 2013; Kirchner et al., 2000), I cannot exclude that the missing effects of tVNS in Studies 2 and 4 may have been related to a non-effective application of tVNS. This methodological issue requires more research on the basic mechanisms as well as stimulation parameters and the development of a reliable test for the methodological correctness of the tVNS application in healthy participants. One possible study design could be adapted from Janner et al. (2018). In a within-subject design, mood-changing effects of tVNS could be assessed on arousal measures such as HR, sAA and electrodermal

activity. Moreover, it is still unclear, whether the sham stimulation does not impact the activity of the vagus nerve. Therefore, a systematic investigation for better control conditions would shed light on the effectiveness and physiological manipulation of verum tVNS. In this dissertation, I have applied two different kinds of control stimulations, namely no stimulation, which however might have induced context changes between acquisition and extinction, and sham stimulation at the helix, which might still affect the vagus nerve. One possibility is a sham stimulation on the earlobe (Burger et al., 2017; Burger et al., 2016; Ellrich, 2011; Peuker & Filler, 2002; Ventura-Bort et al., 2018), allowing no context changes. However, the prevention of involuntary vagus stimulation has still systematically to be tested in future research.

Furthermore, studies over several days might have advantages, but also disadvantages. On the one hand, they need a lot of effort, rely a lot on participants' loyalty to return on the following days and are usually more time consuming and costly. On the other hand, studies extending over several days mostly allow memory consolidation overnight, which is very important for learning, and are a closer model to clinical cases. Thus, after having experienced a traumatic event, individuals need several days for developing PTSD and have several "consolidation-nights" after the threatening event until they get exposure therapy.

In an explorative anxiety conditioning study, we extended the protocol to five days, during which the first day included habituation and anxiety acquisition, the second day extinction with prolonged tVNS or sham stimulation similar to Study 4. The third and fourth sessions on Day 3 and Day 4 only contained a stimulation period of 30 min each. After another 30 min stimulation on Day 5, reinstatement, as well as a contextual generalization test similar to the contexts in Study 4, was performed. Importantly, I assessed salivary alpha-amylase samples on each day prior and post experiment. Since

the study sample consisted of only 8 participants for the tVNS group and 8 for the sham group, the preliminary results should be taken with great caution. However, the sAA levels revealed a slight increase in the tVNS compared to the sham group, but only after Day 4, which suggests that in the studies of this dissertation I might not have stimulated enough.

Although few studies revealed activation of the amygdala by tVNS (Frangos et al., 2015), future fMRI studies should compare the activated brain regions in a group of patients, who wear an implanted VNS, with the activated brain regions in a tVNS group. At best, the stimulator would be newly implanted so that permanent changes in body physiology induced by the VNS device can be excluded. Then again, a study design for several days or even weeks would generate the most reliable and controlled VNS/tVNS results which are highly required to interpret the results of all the existing tVNS studies in humans including data of current thesis.

One well-known problem in differential conditioning studies is contingency awareness (Jovanovic et al., 2006; see also Lonsdorf et al., 2017; Weike, Schupp & Hamm, 2007). Without specific instructions about CS-US association, some participants do not (explicitly) learn these associations. As a consequence, the question is how to handle those participants, who on the one hand have been part of data acquisition and are therefore important for statistical power, but on the other hand, they did not perform the learning task appropriately. Interestingly, previous studies reported no necessity of explicit awareness for implicit fear responses (Hamm et al., 2003; Weike et al., 2007), whereas others found the crucial role of contingency awareness for physiological fear acquisition (Lovibond & Shanks, 2002; Tabbert et al., 2011). I here decided for separate analyses with and without the unaware participants, who after the acquisition were not able to report the correct CS–US or CTX–US contingencies. In the current set of conditioning studies, I could not find remarkable differences in the results. One option to

reduce unaware participants in the future without explicitly instructing contingencies could be the selection of a more appropriate US. Kredlow, Orr, and Otto (2018) found better CS+ and CS- discrimination when the compound US of a screaming face and a scream was used (see also Lau et al., 2008). In line with this, ecological validity in the kind of US, which was an electric shock in current experiments, seems very low as usually, visitors of an office do not receive electric stimulation. Sperl, Panitz, Hermann, and Mueller (2016) compared the fear acquisition of two groups of participants, one received a mildly painful electric stimulus, the other heard a 95 dB white noise burst. Though the contingency awareness did not differ between electric painful US and loud noise US groups, they reported more stable fear acquisition in terms of valence and arousal ratings, skin conductance responses and heart rate in the latter group. A less artificial stimulus in an office environment might have been a scream (Kredlow et al., 2018). An alternative would, for example, be the use of the boss's loud and angry voice. Moreover, the cues in the contexts, which were implemented by colored lights, were not likely to appear in an office in real. In future studies, ringing phones with different ring tones or different voices speaking a voice mail might be an alternative cue, i.e. a physic element in the context of an office (Rudy, 2009).

6.7 Conclusions

The goal of this thesis was first the investigation of fear and anxiety acquisition regarding the dual context representation, and second to improve contextual extinction learning and reduce the return of anxiety by transcutaneous vagus nerve stimulation. I can conclude that contexts are represented in an elemental and a conjunctive view in the brain, which both are highly connected but differently reflected on the subjective and electro-cortical level. Moreover, successful experimental anxiety acquisition, extinction,

and reinstatement of conditioned anxiety were implemented in the study designs. However, accelerated extinction and attenuated return of conditioned responses due to tVNS could not be detected. In the future, a better manipulation check for the effectiveness of tVNS and the optimal adjustments in stimulation parameters have to be investigated to further elaborate the very promising memory-enhancing effects of vagus nerve stimulation.

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8 Appendix

a. Determination of the individual electric pain threshold

Untersuchung:

Datum: **VP-Code:**

Schmerzschwellenbestimmung – Intensität

	Serie1-Ansteigen	Serie1-Absteigen	Serie2-Ansteigen	Serie2 -Absteigen
8 mA				
7,5 mA				
7 mA				
6,5 mA				
6 mA				
5,5 mA				
5 mA				
4,5 mA				
4,0 mA				
3,5 mA				
3 mA				
2,5 mA				
2 mA				
1,5 mA				
1 mA				
0,5 mA				
0 mA				

Mittelwert der Intensität (gerundet): _____

+ 30% (x 1,3) _____

Rating Schmerzschwelle: _____

b. Study 1: Telephone interview

„Das Daumenkino-Experiment: Differenzierung zwischen Cue- und Kontextkonditionierung“

Teilnehmer-Code: _____ Datum:_____

Telefonische Vorbefragung

(Ein- Ausschlusskriterien)

1. Wie viele Gläser Alkohol trinken Sie pro Woche? Menge:_____
Weniger als 15 Gläser Alkohol pro Woche: □ ja □ nein

2. Wie viele Zigaretten rauchen Sie täglich? Menge:_____
Nicht mehr als 20 Zigaretten pro Tag: □ ja □ nein

3. Konsumieren Sie illegale Drogen: □ ja □ nein

4. Nehmen Sie regelmäßig verschreibungspflichtige Medikamente ein?: □ ja □ nein
Falls ja: Welche?_____
Kontraindikation: Zentral wirksame Medikamente, z.B. Neuroleptika, Antidepressiva, Antiepileptika, Opiate, Benzodiazepine

5. Leiden Sie an einer psychischen Erkrankung (Angststörungen, Depression, Schizophrenie, Alkohol-, Drogen-, Medikamentenabhängigkeit? □ ja □ nein
Falls ja: Welche?_____
isolierte Phobien (z.B. Spinnen, Spritzen) auch ausschließen!

6. Leiden Sie an einer neurologischen Erkrankung? □ ja □ nein
Falls ja: Welche?_____
Kontraindikation: Erkrankungen mit Beteiligung des ZNS, z.B. Schlaganfall, Gehirnblutungen, Epilepsie, Parkinson, MS

7. Leiden Sie an einer sonstigen Erkrankung (Herz-Kreislauf, Blut, Lunge, Leber, Nieren, Schilddrüse, Augen, Haut, Magen-Darmtrakt, Stoffwechsel): □ ja □ nein
Falls ja: Welche?_____
Kontraindikation: schwere Erkrankungen

8. Wird Ihnen während Karussell-, Schiffs- oder Flugzeugfahrten schnell schwindelig oder übel? □ ja □ nein

9. Sind Sie farbenblind? □ ja □ nein

10. Leiden Sie unter Hörproblemen? □ ja □ nein

„Das Daumenkino-Experiment: Differenzierung zwischen Cue- und Kontextkonditionierung“

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11. Tragen Sie einen Herzschrittmacher oder eine Insulinpumpe? Ja Nein

12. Leiden Sie an epileptischen Anfällen ? Ja Nein

13. Besteht eine Schwangerschaft ? Ja Nein

14. Nehmen Sie die Pille? Ja Nein

Termin VR-Experiment: _____

c. Study 1: Study Information

Julius-Maximilians-
UNIVERSITÄT
WÜRZBURG

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B. Sc. Katrin Freundorfer
E-Mail: katrin.freundorfer@stud-mail.uni-wuerzbuerg.de

Würzburg, Juli 2015

Probandeninformation zur Studie

„Das Daumenkino-Experiment: Differenzierung zwischen Cue- und Kontextkonditionierung“

Teilprojekt B01
im Rahmen des SFB Transregio 58 Furcht, Angst, Angsterkrankungen

Sehr geehrte Versuchsteilnehmerin, sehr geehrter Versuchsteilnehmer,

Sie haben Gelegenheit, an einer von der Deutschen Forschungsgemeinschaft geförderten Studie teilzunehmen, mit der wir untersuchen wollen, unter welchen Bedingungen bestimmte Gegenstände oder Umwelten unangenehme Gefühle (z. B. Angst) auslösen. Sie werden aus der Teilnahme keinen unmittelbaren Nutzen für sich ziehen können. Wir hoffen jedoch, durch unsere Arbeit mehr darüber zu erfahren, wie Angststörungen entstehen und welche Bedingungen sie aufrechterhalten, um so langfristig die Behandlung zu verbessern.

Vor der Untersuchung werden Sie einige Fragebögen ausfüllen, in denen wichtige Daten bezüglich Ihrer Person festgehalten werden. Dann wird die Versuchsleiterin zur Messung der elektro-kortikalen Potenziale Ihres Gehirns mehrere Messelektroden in Ihrem Gesicht und auf Ihrem Kopf anbringen. Dazu wird Ihre Haut mit Alkohol und einer Peelingpaste gereinigt, um den elektrischen Widerstand zwischen Haut und Messelektrode so gering wie möglich zu halten. Aufgrund dieser Hautreinigung kann es zu Hautrötungen oder leichten Hautirritationen kommen, die aber normalerweise innerhalb kurzer Zeit abklingen.

Während der Untersuchung werden wir Ihnen Bilder einer virtuelle Welt, d. h. von einem Computer erzeugte Räume, auf einem großen Bildschirm (Powerwall) vor Ihnen zeigen. Sie sollen diese Räume und die darin enthaltenen Gegenstände aufmerksam betrachten. In seltenen Fällen kann die virtuelle Realität Übelkeit oder Schwindel auslösen, ähnlich wie eine Karussellfahrt. Falls dies passiert, so teilen Sie uns das bitte sofort mit.

Manchmal werden Sie elektrische Reize am Unterarm verspüren. Diese elektrischen Reize sind etwas schmerzhaft, aber sehr kurz und nicht gefährlich. Die Stärke der elektrischen Reize wird individuell ermittelt und vor Versuchsbeginn festgelegt.

Damit Sie sich den Untersuchungsablauf und die darin vorkommenden virtuellen Welten und elektrischen Reize besser vorstellen können, werden wir Ihnen zu Beginn der Untersuchung jeweils Beispiele dafür präsentieren.

Alle Daten dienen ausschließlich Forschungszwecken, werden vertraulich behandelt und ohne Namensgebung unter einer Codenummer abgespeichert (Pseudonymisierung). Der Codierungsschlüssel, der die Zuordnung Ihres Namens zu der Codenummer erlaubt, wird ein Jahr nach Abschluss der Studie vernichtet (Anonymisierung). Bis dahin können Sie die Löschung Ihrer Daten verlangen. Die anonymisierten Daten werden ansonsten für unbestimmte Zeit gespeichert.

Die hier erhobenen Daten dienen rein wissenschaftlichen Zwecken und werden ohne Bezug auf konkrete Personen ausgewertet und in wissenschaftlichen Fachzeitschriften veröffentlicht.

Die Teilnahme an der Untersuchung ist völlig freiwillig. Sie können jederzeit - ohne Angabe von Gründen - die Teilnahme abbrechen. Dadurch entstehen Ihnen keinerlei persönliche Nachteile.

Falls Sie noch weitere Fragen haben, stellen Sie diese bitte jetzt.

d. Study 1: Informed consent

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E-Mail: katrin.freundorfer@stud-mail.uni-wuerzburg.de

Würzburg, Juli 2015

Einverständniserklärung zur Datenerhebung im Rahmen der Studie

„Das Daumenkino-Experiment: Differenzierung zwischen Cue- und Kontextkonditionierung“

Teilprojekt B01
im Rahmen des SFB Transregio 58 Furcht, Angst, Angsterkrankungen

Durch meine Unterschrift bestätige ich:

Ich nehme freiwillig an der Untersuchung „Differenzierung zwischen Cue- und Kontextkonditionierung“ teil und bin damit einverstanden, dass die erhobenen Daten wissenschaftlich ausgewertet werden. Ich bin auch damit einverstanden, dass die Ergebnisse der Studie, in Gruppen zusammengefasst, wissenschaftlich veröffentlicht werden.

Über mögliche Risiken wurde ich aufgeklärt. Ich weiß auch, dass es nicht möglich ist, Informationen über individuelle Untersuchungsergebnisse (z.B. persönliche Risikokonstellationen) zu erhalten.

Ich hatte ausreichend Zeit, mir zu überlegen, ob ich an der Datenerhebung teilnehmen will, sowie Gelegenheit, Fragen zu stellen. Mit den erhaltenen Antworten bin ich zufrieden. Ich habe darüber hinaus eine Probandeninformation und eine Kopie dieser Einverständniserklärung (datiert und unterschrieben) erhalten. Ich wurde darauf hingewiesen, dass ich jederzeit und ohne Angaben von Gründen von dieser Untersuchung zurücktreten kann, ohne dass mir dadurch ein Nachteil entsteht. Die Daten werden in diesem Falle vernichtet. Ich kann auch nach der Teilnahme an dieser Studie die Löschung der hier erhobenen Daten verlangen. Ein Jahr nach Abschluss der Studie wird aber der Codierungsschlüssel gelöscht und damit ist die Zuordnung meines Namens zu meinen hier erhobenen Daten (und damit auch die Löschung der Daten) nicht mehr möglich.

Name des Teilnehmers / der Teilnehmerin:.....
(bitte Blockbuchstaben)

....
Ort, Datum Unterschrift Teilnehmer/in

.....
Unterschrift aufklärende/r Mitarbeiter/in

e. Study 1: Instructions of the pain scale

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Lehrstuhl für Psychologie I - Prof. Dr. Paul Pauli
Biologische Psychologie, Klinische Psychologie und Psychotherapie

Instruktion zur Studie

„Das Daumenkino-Experiment: Differenzierung zwischen Cue- und Kontextkonditionierung“

Teilprojekt B01
im Rahmen des SFB Transregio 58 Furcht, Angst, Angsterkrankungen

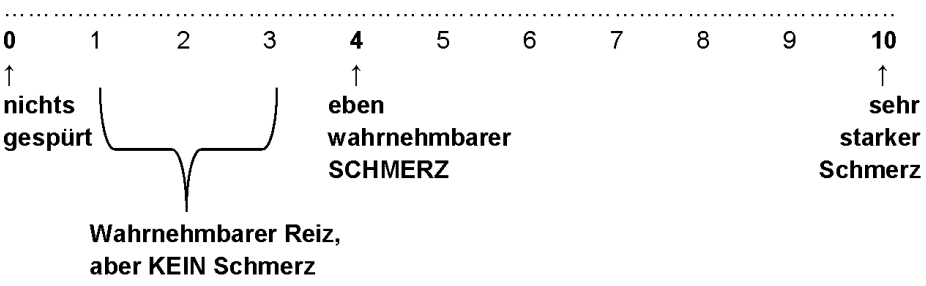
Sehr geehrte Versuchsteilnehmerin, sehr geehrter Versuchsteilnehmer,

Vielen Dank, dass Sie sich bereit erklärt haben, an unserem Experiment teilzunehmen.

Zuerst wird Ihre persönliche Schmerzschwelle bestimmt. Zum einen können wir so die auf Sie passende Reizintensität auswählen, zum anderen können Sie die Reize, die Sie im Experiment erhalten werden, kennenlernen. Über eine Elektrode an Ihrem Unterarm werden Sie dann verschieden starke Reize erhalten. Manche Reize sind eventuell so schwach, dass Sie diese gar nicht spüren können. Ihre Aufgabe ist es, dem Versuchsleiter nach jedem Reiz mitzuteilen, wie stark dieser war.

Dazu wird Ihnen folgende Frage gestellt:

Wie stark war dieser elektrische Reiz auf der Skala von 0 bis 10?



Bitte beachten Sie:
0 bedeutet „**nichts gespürt**“,
1-3 bedeuten **„etwas gespürt, aber NICHT schmerzhaft“**,
4 bedeutet „eben wahrnehmbarer **SCHMERZ**“, d.h. ab hier schmerzhaft!

Bitte prägen Sie sich diese Skala gut ein. Sie werden nach jedem Reiz mündlich aufgefordert, den Reiz zu beurteilen.

f. Study 1: Demographic questionnaire

Testdatum: _____ Vp-Nr.: _____

V O R B E F R A G U N G

Wir möchten Sie bitten, einige Angaben zu ihrer Person zu machen. Diese sind notwendig, da individuelle Faktoren (wie z.B. Ihr Alter) einen Einfluss auf die Testergebnisse haben könnten
Sie können sich darauf verlassen, dass diese streng vertraulich bleiben.

1. Alter: : ……..

2. Geschlecht: männlich …… ☐
weiblich …… ☐

3. Familienstand: ledig ..... ☐
verheiratet ..... ☐
in Lebensgemeinschaft lebend ..... ☐
geschieden ..... ☐
getrennt lebend ..... ☐
verwitwet ..... ☐

4. Studienfach: …… Semesterzahl: …..……

5. Händigkeit : ☐ rechts ☐ links ☐ beidhändig

6. Haben Sie eine Sehschwäche? ☐ ja ☐ nein

Wird Ihre Sehfähigkeit ausreichend korrigiert? ☐ ja ☐ nein

7. Leiden Sie an einer psychiatrischen oder neurologischen Erkrankung (wenn ja, welche)?

_____

8. Hatten Sie schon einmal einen epileptischen Anfall oder ist bei Ihnen in der Familie eine Epilepsieerkrankung bekannt? ☐ ja ☐ nein

9. Nehmen Sie regelmäßig oder zurzeit Medikamente (welche, wie oft)?

_____

10. nur weibliche Versuchsteilnehmer: verwenden Sie hormonelle Verhütungsmittel?

Wenn ja: Was? (Art und Name/Marke)

Der wievielte Tag seit dem ersten Tag Ihrer letzten Perioden ist heute?

g. Study 2: Telephone interview before participants were invited

Die Effekte transkutaner Vagusnervstimulation auf das Extinktionslernen nach Furchtkonditionierung

Teilnehmer-Code: _____ Datum:_____

Telefonische Vorbefragung

(Ein- Ausschlusskriterien)

1. Wie viele Gläser Alkohol trinken Sie pro Woche? Menge:_____
Weniger als 15 Gläser Alkohol pro Woche: □ ja □ nein

2. Wie viele Zigaretten rauchen Sie täglich? Menge:_____
Nicht mehr als 20 Zigaretten pro Tag: □ ja □ nein

3. Konsumieren Sie illegale Drogen: □ ja □ nein

4. Nehmen Sie regelmäßig verschreibungspflichtige Medikamente ein?: □ ja □ nein
Falls ja: Welche?_____
Kontraindikation: Zentral wirksame Medikamente, z.B. Neuroleptika, Antidepressiva, Antiepileptika, Opiate, Benzodiazepine

5. Leiden Sie an einer psychischen Erkrankung (Angststörungen, Depression, Schizophrenie, Alkohol-, Drogen-, Medikamentenabhängigkeit? □ ja □ nein
Falls ja: Welche?_____
isolierte Phobien (z.B. Spinnen, Spritzen) auch ausschließen!

6. Leiden Sie an einer neurologischen Erkrankung? □ ja □ nein
Falls ja: Welche?_____
Kontraindikation: Erkrankungen mit Beteiligung des ZNS, z.B. Schlaganfall, Gehirnblutungen, Epilepsie, Parkinson, MS

7. Leiden Sie an einer sonstigen Erkrankung (Herz-Kreislauf, Blut, Lunge, Leber, Nieren, Schilddrüse, Augen, Haut, Magen-Darmtrakt, Stoffwechsel): □ ja □ nein
Falls ja: Welche?_____
Kontraindikation: schwere Erkrankungen

8. Wird Ihnen während Karussell-, Schiffs- oder Flugzeugfahrten schnell schwindelig oder übel? □ ja □ nein

9. Sind Sie farbenblind? □ ja □ nein

10. Leiden Sie unter Hörproblemen? □ ja □ nein

11. Tragen Sie einen Herzschrittmacher oder eine Insulinpumpe? Ja Nein

12. Leiden Sie an epileptischen Anfällen ? Ja Nein

13. Sind Sie am Herz oder Kopf operiert worden ? Ja Nein

14. Tragen Sie eine Zahnprothese ? Ja Nein

15. Befinden sich in oder auf Ihrem Körper Metallteile/-partikel ? Ja Nein
(z. B. Prothesen, OP-Clips, Splitter, Verhütungsspirale, Tätowierungen, Piercings, "Glitzer-Make-Up" o.ä.)

16. Könnten Fremdkörper im Auge sein ? Ja Nein

17. Besteht eine Schwangerschaft ? Ja Nein

18. Nehmen Sie die Pille? Ja Nein

19. Haben oder hatten Sie beruflich mit Metallverarbeitung zu tun ? Ja Nein

20. Haben Sie einen VNS schonmal gesehen oder benutzt? Ja Nein

21. Haben Sie schon einmal an einer Studie im Powerwall-Labor teilgenommen, in der Büroräume gezeigt wurden? Ja Nein

Termin VR-Experiment: _____

h. Study 2: Demographic questionnaire

Testdatum: _____ Vp-Nr.: _____

V O R B E F R A G U N G

Wir möchten Sie bitten, einige Angaben zu ihrer Person zu machen. Diese sind notwendig, da individuelle Faktoren (wie z.B. Ihr Alter) einen Einfluss auf die Testergebnisse haben könnten
Sie können sich darauf verlassen, dass diese streng vertraulich bleiben.

1. Alter: : ……..

2. Geschlecht: männlich …… ☐
weiblich …… ☐

3. Familienstand: ledig ..... ☐
verheiratet ..... ☐
in Lebensgemeinschaft lebend ..... ☐
geschieden ..... ☐
getrennt lebend ..... ☐
verwitwet ..... ☐

4. Studienfach: …… Semesterzahl: …..……

5. Händigkeit : ☐ rechts ☐ links ☐ beidhändig

6. Haben Sie eine Sehschwäche? ☐ ja ☐ nein

Wird Ihre Sehfähigkeit ausreichend korrigiert? ☐ ja ☐ nein

7. Leiden Sie an einer psychiatrischen oder neurologischen Erkrankung (wenn ja, welche)?

_____

8. Hatten Sie schon einmal einen epileptischen Anfall oder ist bei Ihnen in der Familie eine Epilepsieerkrankung bekannt? ☐ ja ☐ nein

9. Nehmen Sie regelmäßig oder zurzeit Medikamente (welche, wie oft)?

_____

i. Study 2: Study Instructions Day 1 for tVNS and sham group

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Probandeninformation zur Studie VNS-1

„Der Effekt von transkutaner Vagusnervstimulation auf das Extinktionslernen nach Furchtkonditionierung"

Sehr geehrte Versuchsteilnehmerin, sehr geehrter Versuchsteilnehmer,

vielen Dank für Ihr Interesse an unserer Studie!

Ziel dieses Experiments ist die Untersuchung der Wirkung von transkutaner Vagusnervstimulation (= über die Haut) auf die Verarbeitung aversiver, also unangenehmer Reize (z. B. ein elektrischer Reiz). Die Studie wird an 3 aufeinander folgenden Tagen stattfinden. Der Ablauf ist dabei an jedem Tag ähnlich. Wir erhoffen uns durch diese Studie Aufschluss über die Wirkung der Vagusnervstimulation auf die physiologische und subjektive Verarbeitung von emotionalen Reizen. Wenn Sie möchten, werden wir Ihnen nach der Untersuchung gerne die Hintergründe und Ziele dieser Untersuchung ausführlich schildern.

Der Vagusnervstimulator:

Die Firma cerbomed GmbH hat einen Stimulator entwickelt, der einen Ast des Vagusnervs über die Haut am Ohr stimulieren kann. Der Vagusnerv bildet die Verbindung von wichtigen Organen des menschlichen Körpers wie Lunge und Herz zum Gehirn. Je nach Zustand des Körpers (z. B. Stress) wird auf natürliche Weise der Vagusnerv aktiviert und sendet Signale direkt ins Gehirn. Durch die künstliche Stimulation des Vagusnervs können wir von außen über die Haut bestimmte Regionen im Gehirn stimulieren. Sie werden dabei nur ein Kribbeln am Ohr spüren.

Ursprünglich wurde das Gerät für Epilepsiepatienten entwickelt. Sie stimulieren ihren Vagusnerv 4 Stunden pro Tag, um epileptische Anfälle zu unterdrücken. Wir möchten die Vagusnervstimulation auch in der Angsttherapie zum Einsatz bringen. Dieses Projekt soll langfristig Aufschluss über die Effekte der Vagusnervstimulation auf die Angstlöschung geben.

Ist mit irgendwelchen Nebenwirkungen zu rechnen?

Die Anwendung des cerbomed-Stimulators gilt als sehr nebenwirkungsarm. Dennoch können Juckreiz, Empfindungsstörungen im Bereich der äußeren Ohrmuschel und lokale Schmerzen am Stimulationsort nicht ausgeschlossen werden. Jedoch klingen die Nebenwirkungen normalerweise nach Beendigung der Stimulation rasch ab.
An allen Stellen am Körper, an denen Elektroden platziert und Stimulationen appliziert werden, kann es zu einer vorübergehenden Rötung der entsprechenden Stelle führen.

Der Versuchsablauf – Tag 1:

Vor der Untersuchung werden Sie gebeten einige Fragebögen auszufüllen, in denen wichtige Daten bezüglich Ihrer Person und bezüglich Ihres individuellen Angstempfindens sowie Ihrer Ängstlichkeit festgehalten werden. Die Beantwortung der Fragebögen wird ca. 15 Minuten in Anspruch nehmen.

Danach erfolgt ein Test des Vagusnervstimulators. Zwar wird an Tag 1 des Experiments der Stimulator nicht im Versuch integriert sein, jedoch möchten wir gerne, dass Sie das Kribbeln am Ohr kennenlernen und wissen wie sich die Stimulation anfühlt. Sie können sich daraufhin entscheiden, ob Sie am gesamten Experiment teilnehmen möchten, oder nur am ersten Tag ohne die Stimulation.

Dann wird der Versuchsleiter zur Messung Ihrer Schweißdrüsenaktivität, Ihrer Muskelaktivität und Ihres EKGs mehrere Messelektroden auf Ihrer Hand und Ihrem Körper anbringen. Dazu wird Ihre Haut mit Alkohol gereinigt, um den elektrischen Widerstand zwischen Haut und Messelektrode so gering wie möglich zu halten. Aufgrund dieser Hautreinigung kann es zu Hautrötungen oder leichten Hautirritationen kommen, die aber normalerweise innerhalb kurzer Zeit abklingen.

Außerdem werden Ihnen elektrische Reize am Unterarm appliziert. Dafür wird eine Elektrode an Ihrem Unterarm angebracht. Die Reize sollen leicht schmerzhaft sein. Um die Reize Ihrer persönlichen Schmerzschwelle anzupassen, werden wir vor dem Experiment eine Schwellenbestimmung durchführen. Dabei geben wir Ihnen mehrere elektrische Reize, die Sie dann jeweils auf einer Skala von *„nichts gespürt"* bis *„extrem schmerzhaft"* beurteilen sollen. Aus Ihren Angaben errechnen wir Ihre individuelle Schmerzschwelle. Die Reize während des Experiments orientieren sich dann an genau dieser ermittelten Reizstärke.

Im Experiment werden Sie außerdem über Kopfhörer ein kurzes, lautes Geräusch hören. Dieses Geräusch wird unangenehm für Sie sein und Sie werden sich erschrecken. Es ist aber unschädlich und wichtig für die physiologischen Messungen. Bitte lassen Sie sich dadurch nicht stören.

Während der Untersuchung werden wir Ihnen eine Virtuelle Welt, d. h. von einem Computer erzeugte Räume, zeigen. Sie sollen diese Räume und die darin enthaltenen Gegenstände aufmerksam betrachten. In seltenen Fällen kann die Virtuelle Realität Übelkeit oder Schwindel auslösen, ähnlich wie eine Karussellfahrt. Falls dies passiert, so teilen Sie uns das bitte sofort mit.

Nach Anbringung der Elektroden und Messgeräte wird die Schwellenbestimmung für die elektrischen Reize durchgeführt. Dann kann das Experiment beginnen.

Damit Sie sich den Untersuchungsablauf und die darin vorkommenden Virtuellen Welten, elektrischen Reize und Geräusche besser vorstellen können, werden wir Ihnen zu Beginn der Untersuchung jeweils Beispiele dafür präsentieren.

Sie werden nun mehrmals die virtuellen Räume sehen und laufen auf aufgenommenen Pfaden durch die Zimmer. Dabei bekommen Sie manchmal elektrische Reize appliziert. Zusätzlich wird gelegentlich Ihr Empfinden abgefragt.

Das gesamte Experiment wird ca. 2 Stunden dauern.

Datenschutz

Alle erhobenen Daten dienen ausschließlich Forschungszwecken, werden vertraulich behandelt und ohne Namensgebung unter einer Codenummer abgespeichert (Pseudonymisierung). Der Codierungsschlüssel, der die Zuordnung Ihres Namens zu der Codenummer erlaubt, wird ein Jahr nach Abschluss der Studie vernichtet (Anonymisierung). Bis dahin können Sie die Löschung Ihrer Daten verlangen. Ansonsten werden die anonymisierten Daten für unbestimmte Zeit gespeichert.
Die hier erhobenen Daten dienen rein wissenschaftlichen Zwecken und werden ohne Bezug auf konkrete Personen ausgewertet. Individuelle Studienergebnisse können somit nicht an die Probanden weitergegeben werden. Im Falle einer Veröffentlichung der Daten in wissenschaftlichen Fachzeitschriften werden die Ergebnisse als Gesamtheit der Stichprobe dargestellt.

Falls Sie unter Ängsten leiden:

Ein spezifischer therapeutischer Effekt ist durch die Teilnahme an dieser Studie nicht zu erwarten. Sie können aber zusätzlich nach der Untersuchung eine Beratung bezüglich der Möglichkeiten einer besseren Bewältigung Ihrer Angst in Anspruch nehmen. Eine Weitervermittlung zu einem erfahrenen Psychotherapeuten wird auf Ihren Wunsch in die Wege geleitet.

Die Teilnahme an der Untersuchung ist völlig freiwillig. Sie können jederzeit - ohne Angabe von Gründen - die Teilnahme abbrechen. Dadurch entstehen Ihnen keinerlei persönliche Nachteile.

Falls Sie noch weitere Fragen haben, stellen Sie diese bitte jetzt.

Versuchsleiter:

M. Sc. Hannah Genheimer

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j. Study 2: Study Instructions Day 1 for control group

Julius-Maximilians-
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Lehrstuhl für Psychologie I - Prof. Dr. Paul Pauli
Biologische Psychologie, Klinische Psychologie und Psychotherapie

Lehrstuhl für Psychologie I, Marcusstr. 9-11, 97070 Würzburg

Probandeninformation zur Studie VNS-1

„Der Effekt von transkutaner Vagusnervstimulation auf das Extinktionslernen nach Furchtkonditionierung"

Sehr geehrte Versuchsteilnehmerin, sehr geehrter Versuchsteilnehmer,

vielen Dank für Ihr Interesse an unserer Studie!

Ziel dieses Experiments ist die Untersuchung der Wirkung von transkutaner Vagusnervstimulation (= über die Haut) auf die Verarbeitung aversiver, also unangenehmer Reize (z. B. ein elektrischer Reiz). Die Studie wird an 3 aufeinander folgenden Tagen stattfinden. Der Ablauf ist dabei an jedem Tag ähnlich. Wir erhoffen uns durch diese Studie Aufschluss über die Wirkung der Vagusnervstimulation auf die physiologische und subjektive Verarbeitung von emotionalen Reizen. Wenn Sie möchten, werden wir Ihnen nach der Untersuchung gerne die Hintergründe und Ziele dieser Untersuchung ausführlich schildern.

Der Vagusnervstimulator:

Die Firma cerbomed GmbH hat einen Stimulator entwickelt, der einen Ast des Vagusnervs über die Haut am Ohr stimulieren kann. Der Vagusnerv bildet die Verbindung von wichtigen Organen des menschlichen Körpers wie Lunge und Herz zum Gehirn. Je nach Zustand des Körpers (z. B. Stress) wird auf natürliche Weise der Vagusnerv aktiviert und sendet Signale direkt ins Gehirn. Durch die künstliche Stimulation des Vagusnervs können wir von außen über die Haut bestimmte Regionen im Gehirn stimulieren.

Ursprünglich wurde das Gerät für Epilepsiepatienten entwickelt. Sie stimulieren ihren Vagusnerv 4 Stunden pro Tag, um epileptische Anfälle zu unterdrücken. Wir möchten die Vagusnervstimulation auch in der Angsttherapie zum Einsatz bringen. Dieses Projekt soll langfristig Aufschluss über die Effekte der Vagusnervstimulation auf die Angstlöschung geben.

Ist mit irgendwelchen Nebenwirkungen zu rechnen?

Die Anwendung des cerbomed-Stimulators gilt als sehr nebenwirkungsarm. Dennoch können Juckreiz, Empfindungsstörungen im Bereich der äußeren Ohrmuschel und lokale Schmerzen am Stimulationsort nicht ausgeschlossen werden. Jedoch klingen die Nebenwirkungen normalerweise nach Beendigung der Stimulation rasch ab.
An allen Stellen am Körper, an denen Elektroden platziert und Stimulationen appliziert werden, kann es zu einer vorübergehenden Rötung der entsprechenden Stelle führen.

Der Versuchsablauf – Tag 1:

Vor der Untersuchung werden Sie gebeten einige Fragebögen auszufüllen, in denen wichtige Daten bezüglich Ihrer Person und bezüglich Ihres individuellen Angstempfindens sowie Ihrer Ängstlichkeit festgehalten werden. Die Beantwortung der Fragebögen wird ca. 15 Minuten in Anspruch nehmen.

Danach erfolgt ein Test des Vagusnervstimulators. Zwar wird an Tag 1 des Experiments der Stimulator nicht im Versuch integriert sein, jedoch möchten wir gerne, dass Sie wissen wie sich die Stimulation anfühlt. Sie können sich daraufhin entscheiden, ob Sie am gesamten Experiment teilnehmen möchten.

Dann wird der Versuchsleiter zur Messung Ihrer Schweißdrüsenaktivität, Ihrer Muskelaktivität und Ihres EKGs mehrere Messelektroden auf Ihrer Hand und Ihrem Körper anbringen. Dazu wird Ihre Haut mit Alkohol gereinigt, um den elektrischen Widerstand zwischen Haut und Messelektrode so gering wie möglich zu halten. Aufgrund dieser Hautreinigung kann es zu Hautrötungen oder leichten Hautirritationen kommen, die aber normalerweise innerhalb kurzer Zeit abklingen.

Außerdem werden Ihnen elektrische Reize am Unterarm appliziert. Dafür wird eine Elektrode an Ihrem Unterarm angebracht. Die Reize sollen leicht schmerzhaft sein. Um die Reize Ihrer persönlichen Schmerzschwelle anzupassen, werden wir vor dem Experiment eine Schwellenbestimmung durchführen. Dabei geben wir Ihnen mehrere elektrische Reize, die Sie dann jeweils auf einer Skala von *„nichts gespürt"* bis *„extrem schmerzhaft"* beurteilen sollen. Aus Ihren Angaben errechnen wir Ihre individuelle Schmerzschwelle. Die Reize während des Experiments orientieren sich dann an genau dieser ermittelten Reizstärke.

Im Experiment werden Sie außerdem über Kopfhörer ein kurzes, lautes Geräusch hören. Dieses Geräusch wird unangenehm für Sie sein und Sie werden sich erschrecken. Es ist aber unschädlich und wichtig für die physiologischen Messungen. Bitte lassen Sie sich dadurch nicht stören.

Während der Untersuchung werden wir Ihnen eine Virtuelle Welt, d. h. von einem Computer erzeugte Räume, zeigen. Sie sollen diese Räume und die darin enthaltenen Gegenstände aufmerksam betrachten. In seltenen Fällen kann die Virtuelle Realität Übelkeit oder Schwindel auslösen, ähnlich wie eine Karussellfahrt. Falls dies passiert, so teilen Sie uns das bitte sofort mit.

Nach Anbringung der Elektroden und Messgeräte wird die Schwellenbestimmung für die elektrischen Reize durchgeführt. Dann kann das Experiment beginnen.

Damit Sie sich den Untersuchungsablauf und die darin vorkommenden Virtuellen Welten, elektrischen Reize und Geräusche besser vorstellen können, werden wir Ihnen zu Beginn der Untersuchung jeweils Beispiele dafür präsentieren.

Sie werden nun mehrmals die virtuellen Räume sehen und laufen auf aufgenommenen Pfaden durch die Zimmer. Dabei bekommen Sie manchmal elektrische Reize appliziert. Zusätzlich wird gelegentlich Ihr Empfinden abgefragt.

Das gesamte Experiment wird heute ca. 1,5 Stunden dauern.

Datenschutz

Alle erhobenen Daten dienen ausschließlich Forschungszwecken, werden vertraulich behandelt und ohne Namensgebung unter einer Codenummer abgespeichert (Pseudonymisierung). Der Codierungsschlüssel, der die Zuordnung Ihres Namens zu der Codenummer erlaubt, wird ein Jahr nach Abschluss der Studie vernichtet (Anonymisierung). Bis dahin können Sie die Löschung Ihrer Daten verlangen. Ansonsten werden die anonymisierten Daten für unbestimmte Zeit gespeichert.
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Falls Sie unter Ängsten leiden:

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k. Study 2: Informed consent

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Würzburg, 05.04.2019

Einwilligungserklärung zur Datenerhebung im Rahmen des Projekts

„Der Effekt von transkutaner Vagusnervstimulation auf das Extinktionslernen nach Furchtkonditionierung" – VNS-1

Durch meine Unterschrift bestätige ich:

Ich nehme freiwillig an der dreitägigen Untersuchung „Die Wirkung von transkutaner Vagusnervstimulation auf Extinktionslernen" teil und bin damit einverstanden, dass die erhobenen Daten wissenschaftlich ausgewertet werden. Ich bin auch damit einverstanden, dass die Ergebnisse der Studie, in Gruppen zusammengefasst, wissenschaftlich veröffentlicht werden.

Über mögliche Risiken wurde ich aufgeklärt. Ich weiß auch, dass es nicht möglich ist, Informationen über individuelle Untersuchungsergebnisse (z.B. persönliche Risikokonstellationen) zu erhalten.

Ich hatte ausreichend Zeit mir zu überlegen, ob ich an der Datenerhebung teilnehmen will sowie Gelegenheit Fragen zu stellen. Mit den erhaltenen Antworten bin ich zufrieden. Ich habe darüber hinaus eine Probandeninformation und eine Kopie dieser Einwilligungserklärung (datiert und unterschrieben) erhalten. Ich wurde darauf hingewiesen, dass ich jederzeit von dieser Untersuchung zurücktreten kann, ohne dass mir dadurch ein Nachteil entsteht. Die Daten werden in diesem Falle vernichtet. Ich kann auch nach der Teilnahme an dieser Studie die Löschung der hier erhobenen Daten verlangen. Ein Jahr nach Abschluss der Studie wird aber der Codierungsschlüssel gelöscht und damit ist die Zuordnung meines Namens zu meinen hier erhobenen Daten (und damit auch die Löschung der Daten) nicht mehr möglich.

Name des Teilnehmers: ..... (bitte Blockbuchstaben)

....

Ort, Datum Unterschrift des Teilnehmers

Name des aufklärenden Mitarbeiters: .....

....

Ort, Datum Unterschrift des aufklärenden Mitarbeiters

I. Study 2: Instructions of the rating scales

Lehrstuhl für Psychologie I - Prof. Dr. Paul Pauli
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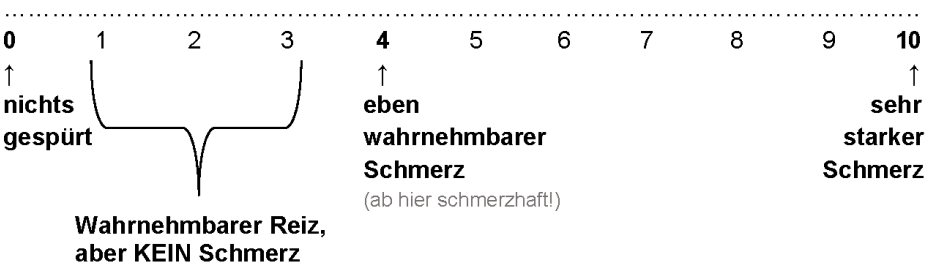
Instruktion zur Studie

Zuerst wird Ihre persönliche Schmerzschwelle bestimmt.
Ihre Aufgabe ist es, dem Versuchsleiter nach jedem elektrischen Reiz mitzuteilen, wie stark dieser war.

Dazu wird Ihnen folgende Frage gestellt:

Wie stark war dieser elektrische Reiz auf der Skala von 0 bis 10?



Nach aktiver Erkundung der virtuellen Welt, werden sie anschließend mehrmals passiv durch die verschiedenen Räume geführt. Danach werden Ihnen Ausschnitte aus den zuvor gesehenen Räumen präsentiert und einige Fragen zu Ihren Empfindungen darin gestellt, die Sie mit Hilfe der folgenden Skalen beantworten sollen.

Wie positiv oder negativ empfanden Sie diesen Raum?

Nennen Sie dann bitte eine Zahl von **0 (sehr negativ)** bis **100 (sehr positiv)** auf der unten angegebenen Skala.

0 50 **100**

Wie stark war Ihre Aufregung in diesem Raum?

Nennen Sie dann bitte eine Zahl von **0 (gar keine Aufregung)** bis **100 (sehr starke Aufregung)** auf der unten angegebenen Skala.

.....

0 50 **100**

Wie groß war Ihre Angst in diesem Raum?

Nennen Sie dann bitte eine Zahl von **0 (keine Angst)** bis **100 (sehr starke Angst)** auf der unten angegebenen Skala.

.....

0 50 **100**

Wie sehr haben Sie den elektrischen Reiz in diesem Raum erwartet?

Nennen Sie dann bitte eine Zahl von **0 (gar nicht)** bis **100 (ganz sicher)** auf der unten angegebenen Skala.

.....

0 50 **100**

Bitte prägen Sie sich alle Skalen gut ein. Wenn Sie später danach gefragt werden, antworten Sie bitte mündlich und möglichst spontan und zügig.

Falls Sie noch weitere Fragen haben, stellen Sie diese bitte jetzt.

m. Study 2: Study Instructions Day 2 for tVNS and sham group

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„Der Effekt von transkutaner Vagusnervstimulation auf das Extinktionslernen nach Furchtkonditionierung"

Sehr geehrte Versuchsteilnehmerin, sehr geehrter Versuchsteilnehmer,

herzlich Willkommen zu Versuchstag 2. Schön, dass Sie wieder mitmachen!

Der Versuchsablauf – Tag 2:

Der Versuch läuft genauso ab wie an Tag 1. Sie werden gebeten Fragebögen auszufüllen und dann werden Messelektroden und Geräte an Ihnen angebracht. Zusätzlich wird noch der Vagusnervstimulator wie an Tag 1 getestet in Ihr Ohr gesetzt.

Für eine gute Leitfähigkeit wird auch hier zunächst Ihre Haut mit Alkohol gereinigt. Um die für Sie individuell effektivste Stimulationsintensität zu bestimmen, wird zunächst eine Schwellenbestimmung vorgenommen. Dabei erhalten Sie mehrere Stimulationen unterschiedlicher Reizintensitäten. Nach jedem Reiz werden Sie gebeten die Stimulation auf einer Skala von *„nichts gespürt"* bis *„Schmerz"* zu beurteilen. Aus diesen Intensitäten wird Ihre individuelle Reizintensität für das ganze Experiment festgelegt.
Dann erfolgt eine ca. 20-minütige Vagusnervstimulation. Dabei wechseln sich Stimulations- und Pausenintervalle regelmäßig ab. Sie können sich währenddessen entspannen.

Genau wie an Tag 1 werden Sie danach wieder durch die virtuellen Räume „laufen". Währenddessen wird der Vagusnervstimulator an sein. Es werden Ihnen genau wie am Vortag wieder elektrische Reize verabreicht. Außerdem hören Sie von Zeit zu Zeit wieder das unangenehme, erschreckende Geräusch über die Kopfhörer. Wie an Tag 1 werden Sie zwischendurch gebeten, Einschätzungen zu Ihrem Empfinden zu geben.

Die Untersuchung wird ca. 2 Stunden dauern.

Datenschutz

Alle erhobenen Daten dienen ausschließlich Forschungszwecken, werden vertraulich behandelt und ohne Namensgebung unter einer Codenummer abgespeichert (Pseudonymisierung). Der Codierungsschlüssel, der die Zuordnung Ihres Namens zu der Codenummer erlaubt, wird ein Jahr nach Abschluss der Studie vernichtet (Anonymisierung). Bis dahin können Sie die Löschung Ihrer Daten verlangen. Ansonsten werden die anonymisierten Daten für unbestimmte Zeit gespeichert.
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n. Study 2: Study Instructions Day 2 for control group

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Genau wie an Tag 1 werden Sie danach wieder durch die virtuellen Räume „laufen". Währenddessen wird der Vagusnervstimulator an sein. Es können Ihnen genau wie am Vortag wieder elektrische Reize verabreicht werden. Außerdem hören Sie von Zeit zu Zeit wieder das unangenehme, erschreckende Geräusch über die Kopfhörer. Wie an Tag 1 werden Sie zwischendurch gebeten, Einschätzungen zu Ihrem Empfinden zu geben.

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o. Study 2: Instructions of tVNS rating scale

Lehrstuhl für Psychologie I - Prof. Dr. Paul Pauli
Biologische Psychologie, Klinische Psychologie und Psychotherapie

Instruktion zur Vagusnervstimulation

Als erstes wird Ihre persönliche Stimulationsschwelle bestimmt.
Hierfür wird Ihnen ein Vagusnervstimulator ins Ohr gesetzt und Sie erhalten mehrere Stimulationen unterschiedlicher Reizintensitäten. Nach jedem Reiz werden Sie gebeten die Stimulation auf der folgenden Skala zu beurteilen. Daraus wird im Anschluss Ihre individuelle Reizintensität für das ganze Experiment festgelegt.

Wie empfanden Sie die Stimulation?

……

0	1	2	**3**	4	5	**6**	7	8	9	**10**
↑			↑			↑				↑
nichts gespürt			**leichtes Kribbeln**			**starkes Kribbeln**				**Schmerz**

Nun kann das Experiment beginnen. Sie werden erneut durch die virtuellen Räume geführt, währenddessen wird der Vagusnervstimulator an sein.

Falls Sie noch weitere Fragen haben, wenden Sie sich bitte jederzeit an Ihre Versuchsleiterin.

p. Study 2: Questionnaire on participant's subjective experience with the ear stimulation

Datum: Vp.Nr:

Nachbefragung

Wie gut hat die Vagusnervstimulation bei Ihnen funktioniert?

1-------2-------3-------4-------5-------6-------7-------8-------9-------10

gar nicht gut sehr gut

Wie angenehm war die Vagusnervstimulation für Sie?

1-------2-------3-------4-------5-------6-------7-------8-------9-------10

gar nicht gut sehr gut

Wie sehr glauben Sie an die Vagusnervstimulation?

1-------2-------3-------4-------5-------6-------7-------8-------9-------10

gar nicht gut sehr gut

Was halten Sie von der Vagusnervstimulation?

_____
_____
_____

Können Sie sich vorstellen, dass die Vagusnervstimulation klinisch eingesetzt wird?

_____
_____
_____

q. Study 2: Study Instructions Day 3 for tVNS, sham and control group

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herzlich Willkommen zu Versuchstag 3! Heute findet der vorerst letzte Teil der Studie statt.

Der Versuchsablauf – Tag 3:

Der dritte Untersuchungstag läuft ab wie Tag 1. Der Vagusnerv wird nicht mehr stimuliert.

Sie werden Fragebögen ausfüllen, danach werden die Elektroden wie an den Vortagen angebracht. Auch der Ablauf in virtueller Realität ist gleich.

Dieser Teil des Experiments wird ca. 2 Stunden dauern.

Datenschutz

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r. Study 3: Preliminary interview

Vain-Experiment

Teilnehmer-Code: _____ Datum:_____

Vorbefragung

1. Haben Sie einen Vagusnervstimulator schon einmal gesehen oder benutzt?
□ ja □ nein

2. Wie viele Gläser Alkohol trinken Sie pro Woche? Menge:_____
Weniger als 15 Gläser Alkohol pro Woche: □ ja □ nein

3. Wie viele Zigaretten rauchen Sie täglich? Menge:_____
Nicht mehr als 20 Zigaretten pro Tag: □ ja □ nein

4. Konsumieren Sie illegale Drogen: □ ja □ nein

5. Nehmen Sie regelmäßig verschreibungspflichtige Medikamente ein?: □ ja □ nein
Falls ja: Welche?_____
Kontraindikation: Zentral wirksame Medikamente, z.B. Neuroleptika, Antidepressiva, Antiepileptika, Opiate, Benzodiazepine

6. Leiden Sie an einer psychischen Erkrankung (Angststörungen, Depression, Schizophrenie, Alkohol-, Drogen-, Medikamentenabhängigkeit? □ ja □ nein
Falls ja: Welche?_____
isolierte Phobien (z.B. Spinnen, Spritzen) auch ausschließen!

7. Leiden Sie an einer neurologischen Erkrankung? □ ja □ nein
Falls ja: Welche?_____
Kontraindikation: Erkrankungen mit Beteiligung des ZNS, z.B. Schlaganfall, Gehirnblutungen, Epilepsie, Parkinson, MS

8. Leiden Sie an einer sonstigen Erkrankung (Herz-Kreislauf, Blut, Lunge, Leber, Nieren, Schilddrüse, Augen, Haut, Magen-Darmtrakt, Stoffwechsel): □ ja □ nein
Falls ja: Welche?_____
Kontraindikation: schwere Erkrankungen

9. Sind Sie farbenblind? □ ja □ nein

10. Leiden Sie unter Hörproblemen? □ ja □ nein

11. Tragen Sie einen Herzschrittmacher oder eine Insulinpumpe? Ja Nein

12. Leiden Sie an epileptischen Anfällen ? Ja Nein

13. Besteht eine Schwangerschaft ? Ja Nein

s. Study 3: Study Information for tVNS and sham group

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Lehrstuhl für Psychologie I, Marcusstr. 9-11, 97070 Würzburg

Probandeninformation zur Studie VNS-0

„Die Wirkung von transkutaner Vagusnervstimulation auf Schmerz" im Projekt
„Der Effekt von transkutaner Vagusnervstimulation auf das Extinktionslernen nach Furchtkonditionierung"

Sehr geehrte Versuchsteilnehmerin, sehr geehrter Versuchsteilnehmer,

vielen Dank für Ihr Interesse an unserer Studie!

Ziel des Experiments ist die Untersuchung der Wirkung von transkutaner Vagusnervstimulation (= über die Haut) auf das Schmerzempfinden. Deshalb wird bei Ihnen der Vagusnerv über die Ohrmuschel durch eine kleine elektrische Spannung stimuliert. Sie bekommen verschiedene sensorische Reize wie Druck oder elektrische Reize appliziert und wir möchten dabei gerne Ihre physiologischen Reaktionen (wie Hautleitfähigkeit und EKG) und Ihre subjektive Wahrnehmung der Stimulation messen. Wenn Sie möchten, werden wir Ihnen nach der Untersuchung gerne die Hintergründe und Ziele dieser Untersuchung ausführlich schildern.

Der Vagusnervstimulator:

Die Firma cerbomed GmbH hat einen Stimulator entwickelt, der einen Ast des Vagusnervs über die Haut am Ohr stimulieren kann. Der Vagusnerv bildet die Verbindung von wichtigen Organen des menschlichen Körpers wie Lunge und Herz zum Gehirn. Je nach Zustand des Körpers (z. B. Stress) wird auf natürliche Weise der Vagusnerv aktiviert und sendet Signale direkt ins Gehirn. Durch die künstliche Stimulation des Vagusnervs können wir von außen über die Haut bestimmte Regionen im Gehirn stimulieren. Sie werden dabei nur ein Kribbeln am Ohr spüren.

Ursprünglich wurde das Gerät für Epilepsiepatienten entwickelt. Sie stimulieren ihren Vagusnerv 4 Stunden pro Tag, um epileptische Anfälle zu unterdrücken. Wir möchten die Vagusnervstimulation auch in der Angsttherapie zum Einsatz bringen. Dieses Projekt soll langfristig Aufschluss über die Effekte der Vagusnervstimulation auf die Angstlöschung geben.

Ist mit irgendwelchen Nebenwirkungen zu rechnen?

Die Anwendung des cerbomed-Stimulators gilt als sehr nebenwirkungsarm. Dennoch können Juckreiz, Empfindungsstörungen im Bereich der äußeren Ohrmuschel und lokale Schmerzen am Stimulationsort nicht ausgeschlossen werden. Jedoch klingen die Nebenwirkungen normalerweise nach Beendigung der Stimulation rasch ab.
An allen Stellen am Körper, an denen Elektroden platziert und Stimulationen appliziert werden, kann es zu einer vorübergehenden Rötung der entsprechenden Stelle kommen.

Der Versuchsablauf:

Vor der Untersuchung werden Sie gebeten einige Fragebögen auszufüllen, in denen wichtige Daten bezüglich Ihrer Person festgehalten werden. Die Beantwortung der Fragebögen wird ca. 15 Minuten in Anspruch nehmen.
Dann wird der Versuchsleiter zur Messung Ihrer Schweißdrüsenaktivität und Ihres EKGs mehrere Messelektroden auf Ihrer Hand und Ihrem Körper anbringen. Dazu wird Ihre Haut mit Alkohol gereinigt, um den elektrischen Widerstand zwischen Haut und Messelektrode so gering wie möglich zu halten. Aufgrund dieser Hautreinigung kann es zu Hautrötungen oder leichten Hautirritationen kommen, die aber normalerweise innerhalb kurzer Zeit abklingen.
Der Vagusnervstimulator wird an Ihrem Ohr angebracht. Für eine gute Leitfähigkeit wird auch hier zunächst Ihre Haut mit Alkohol gereinigt. Um die für Sie individuell effektivste Stimulationsintensität zu bestimmen, wird zunächst eine Schwellenbestimmung vorgenommen. Dabei erhalten Sie mehrere Stimulationen unterschiedlicher Reizintensitäten. Nach jedem Reiz werden Sie gebeten die Stimulation auf einer Skala von *„nichts gespürt"* bis *„Schmerz"* zu beurteilen. Aus diesen Intensitäten wird Ihre individuelle Reizintensität für das ganze Experiment festgelegt.
Für die sensorische Stimulation werden Ihnen nacheinander Druckreize und elektrische Reize verabreicht. Die Druckreize erfolgen durch eine stumpfe, dicke Nadel auf der dorsalen Seite der Finger und kann von Ihnen selbst gestartet und gestoppt werden. Der elektrische Reiz wird über eine Elektrode am Unterarm appliziert. Wir bestimmen zunächst ihre individuelle Schmerzschwelle und passen alle Reize während des Experiments daran an. So sind alle Reize an Ihrer persönlichen Wahrnehmung orientiert. Trotzdem haben Sie jederzeit die Möglichkeit die Stimulation und das ganze Experiment abzubrechen.

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Die Teilnahme an der Untersuchung ist völlig freiwillig. Sie können jederzeit - ohne Angabe von Gründen - die Teilnahme abbrechen. Dadurch entstehen Ihnen keinerlei persönliche Nachteile.

Falls Sie noch weitere Fragen haben, stellen Sie diese bitte jetzt.

Versuchsleiter:

M. Sc. Hannah Genheimer

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97070 Würzburg

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e-Mail: hannah.genheimer@uni-wuerzburg.de

t. Study 3: Study Information for control group

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u. Study 3: Informed consent

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Würzburg, 08.04.2019

Einwilligungserklärung zur Datenerhebung im Rahmen des Projekts

„Der Effekt von transkutaner Vagusnervstimulation auf das Extinktionslernen nach Furchtkonditionierung" – VNS-0

Durch meine Unterschrift bestätige ich:

Ich nehme freiwillig an der Untersuchung „Die Wirkung von transkutaner Vagusnervstimulation auf Schmerz" teil und bin damit einverstanden, dass die erhobenen Daten wissenschaftlich ausgewertet werden. Ich bin auch damit einverstanden, dass die Ergebnisse der Studie, in Gruppen zusammengefasst, wissenschaftlich veröffentlicht werden.

Über mögliche Risiken wurde ich aufgeklärt. Ich weiß auch, dass es nicht möglich ist, Informationen über individuelle Untersuchungsergebnisse (z.B. persönliche Risikokonstellationen) zu erhalten.

Ich hatte ausreichend Zeit mir zu überlegen, ob ich an der Datenerhebung teilnehmen will sowie Gelegenheit Fragen zu stellen. Mit den erhaltenen Antworten bin ich zufrieden. Ich habe darüber hinaus eine Probandeninformation und eine Kopie dieser Einwilligungserklärung (datiert und unterschrieben) erhalten. Ich wurde darauf hingewiesen, dass ich jederzeit von dieser Untersuchung zurücktreten kann, ohne dass mir dadurch ein Nachteil entsteht. Die Daten werden in diesem Falle vernichtet. Ich kann auch nach der Teilnahme an dieser Studie die Löschung der hier erhobenen Daten verlangen. Ein Jahr nach Abschluss der Studie wird aber der Codierungsschlüssel gelöscht und damit ist die Zuordnung meines Namens zu meinen hier erhobenen Daten (und damit auch die Löschung der Daten) nicht mehr möglich.

Name des Teilnehmers: ..... (bitte Blockbuchstaben)

....

Ort, Datum Unterschrift des Teilnehmers

Name des aufklärenden Mitarbeiters: .....

....

Ort, Datum Unterschrift des aufklärenden Mitarbeiters

v. Study 3 and 4: Demographic questionnaire

Testdatum: _____ Vp-Nr.: _____

V O R B E F R A G U N G

Wir möchten Sie bitten, einige Angaben zu ihrer Person zu machen. Diese sind notwendig, da individuelle Faktoren (wie z.B. Ihr Alter) einen Einfluss auf die Testergebnisse haben könnten
Sie können sich darauf verlassen, dass diese streng vertraulich bleiben.

1. Alter: : ……..

2. Geschlecht: männlich …… ☐
weiblich …… ☐

3. Familienstand: ledig ..... ☐
verheiratet ..... ☐
in Lebensgemeinschaft lebend ..... ☐
geschieden ..... ☐
getrennt lebend ..... ☐
verwitwet ..... ☐

4. Studienfach: …… Semesterzahl: …..……

5. Händigkeit : ☐ rechts ☐ links ☐ beidhändig

6. Haben Sie eine Sehschwäche? ☐ ja ☐ nein

Wird Ihre Sehfähigkeit ausreichend korrigiert? ☐ ja ☐ nein

7. Leiden Sie an einer psychiatrischen oder neurologischen Erkrankung (wenn ja, welche)?

_____

8. Hatten Sie schon einmal einen epileptischen Anfall oder ist bei Ihnen in der Familie eine Epilepsieerkrankung bekannt? ☐ ja ☐ nein

9. Nehmen Sie regelmäßig oder zurzeit Medikamente (welche, wie oft)?

_____

w. Study 3 and 4: Questionnaire on participant's experience with the ear stimulation

Datum: Vp.Nr:

NACHBEFRAGUNG

Hatten Sie das Gefühl, dass die Vagusnervstimulation bei Ihnen funktioniert hat?

0------1------2------3------4------5------6------7------8------9------10

Nein Ja

Wie angenehm war die Vagusnervstimulation für Sie?

0------1------2------3------4------5------6------7------8------9------10

sehr unangenehm sehr angenehm

Können Sie sich eine Anwendung der Vagusnervstimulation im klinischen Bereich vorstellen?

0------1------2------3------4------5------6------7------8------9------10

Nein Ja

Wie sehr glauben Sie an die Vagusnervstimulation?

0------1------2------3------4------5------6------7------8------9------10

gar nicht sehr gut

Was halten Sie von der Vagusnervstimulation?

_____

_____

Haben Sie während oder nach der Vagusnervstimulation Nebenwirkungen bemerkt?

NEIN ☐ JA ☐

Nebenwirkungen:_____

x. Study 4: Telephone interview

VANX-Experiment

Teilnehmer-Code: _____ Datum:_____

Vorbefragung

1. Haben Sie einen Vagusnervstimulator schon einmal gesehen oder benutzt?
□ ja □ nein

2. Wie viele Gläser Alkohol trinken Sie pro Woche? Menge:_____
Weniger als 15 Gläser Alkohol pro Woche: □ ja □ nein

3. Wie viele Zigaretten rauchen Sie täglich? Menge:_____
Nicht mehr als 20 Zigaretten pro Tag: □ ja □ nein

4. Konsumieren Sie illegale Drogen: □ ja □ nein

5. Nehmen Sie regelmäßig verschreibungspflichtige Medikamente ein?: □ ja □ nein
Falls ja: Welche?_____
Kontraindikation: Zentral wirksame Medikamente, z.B. Neuroleptika, Antidepressiva, Antiepileptika, Opiate, Benzodiazepine

6. Leiden Sie an einer psychischen Erkrankung (Angststörungen, Depression, Schizophrenie, Alkohol-, Drogen-, Medikamentenabhängigkeit? □ ja □ nein
Falls ja: Welche?_____
isolierte Phobien (z.B. Spinnen, Spritzen) auch ausschließen!

7. Leiden Sie an einer neurologischen Erkrankung? □ ja □ nein
Falls ja: Welche?_____
Kontraindikation: Erkrankungen mit Beteiligung des ZNS, z.B. Schlaganfall, Gehirnblutungen, Epilepsie, Parkinson, MS

8. Leiden Sie an einer sonstigen Erkrankung (Herz-Kreislauf, Blut, Lunge, Leber, Nieren, Schilddrüse, Augen, Haut, Magen-Darmtrakt, Stoffwechsel): □ ja □ nein
Falls ja: Welche?_____
Kontraindikation: schwere Erkrankungen

9. Sind Sie farbenblind? □ ja □ nein

10. Leiden Sie unter Hörproblemen? □ ja □ nein

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13. Besteht eine Schwangerschaft ? Ja Nein

y. Study 4: Study information

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Probandeninformation zur Studie VANX

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Der Versuchsablauf:

Das Experiment findet an zwei aufeinanderfolgenden Tagen statt. An beiden Tagen werden Sie vor der Untersuchung gebeten, einige Fragebögen auszufüllen, in denen wichtige Daten bezüglich Ihrer Person festgehalten werden. Die Beantwortung der Fragebögen wird ca. 5 Minuten in Anspruch nehmen.
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Während der Untersuchung werden wir Ihnen eine Virtuelle Welt, d. h. von einem Computer erzeugte Räume, zeigen. Sie sollen diese Räume und die darin enthaltenen Gegenstände aufmerksam betrachten. In seltenen Fällen kann die Virtuelle Realität Übelkeit oder Schwindel auslösen, ähnlich wie eine Karussellfahrt. Falls dies passiert, so teilen Sie uns das bitte sofort mit.
Manchmal werden Sie elektrische Reize am Unterarm verspüren. Diese elektrischen Reize sind etwas schmerzhaft, aber sehr kurz und nicht gefährlich. Die Stärke der elektrischen Reize wird am ersten Tag individuell ermittelt und vor Versuchsbeginn festgelegt.
Während der Untersuchung werden Sie manchmal über Kopfhörer ein kurzes, lautes Geräusch hören. Dieses Geräusch wird unangenehm für Sie sein und Sie werden sich erschrecken. Es ist aber unschädlich und wichtig für die physiologischen Messungen. Bitte lassen Sie sich dadurch nicht stören.
Damit Sie sich den Untersuchungsablauf und die darin vorkommenden Virtuellen Welten, elektrischen Reize und Geräusche besser vorstellen können, werden wir Ihnen zu Beginn der Untersuchung jeweils Beispiele dafür präsentieren.

Datenschutz

Alle erhobenen Daten dienen ausschließlich Forschungszwecken, werden vertraulich behandelt und ohne Namensgebung unter einer Codenummer abgespeichert (Pseudonymisierung). Der Codierungsschlüssel, der die Zuordnung Ihres Namens zu der Codenummer erlaubt, wird ein Jahr nach Abschluss der Studie vernichtet (Anonymisierung). Bis dahin können Sie die Löschung Ihrer Daten verlangen. Ansonsten werden die anonymisierten Daten für unbestimmte Zeit gespeichert.
Die hier erhobenen Daten dienen rein wissenschaftlichen Zwecken und werden ohne Bezug auf konkrete Personen ausgewertet. Individuelle Studienergebnisse können somit nicht an die Probanden weitergegeben werden. Im Falle einer Veröffentlichung der Daten in wissenschaftlichen Fachzeitschriften werden die Ergebnisse als Gesamtheit der Stichprobe dargestellt.

Die Teilnahme an der Untersuchung ist völlig freiwillig. Sie können jederzeit - ohne Angabe von Gründen - die Teilnahme abbrechen. Dadurch entstehen Ihnen keinerlei persönliche Nachteile.

Falls Sie noch weitere Fragen haben, stellen Sie diese bitte jetzt.

Versuchsleiter:

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z. Study 4: Informed consent

Julius-Maximilians-
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Würzburg, 08.04.2019

Einwilligungserklärung zur Datenerhebung im Rahmen des Projekts

„Der Effekt von transkutaner Vagusnervstimulation auf das Extinktionslernen"

Durch meine Unterschrift bestätige ich:

Ich nehme freiwillig an der Untersuchung „Der Effekt von transkutaner Vagusnervstimulation auf das Extinktionslernen" teil und bin damit einverstanden, dass die erhobenen Daten wissenschaftlich ausgewertet werden. Ich bin auch damit einverstanden, dass die Ergebnisse der Studie, in Gruppen zusammengefasst, wissenschaftlich veröffentlicht werden.

Über mögliche Risiken wurde ich aufgeklärt. Ich weiß auch, dass es nicht möglich ist, Informationen über individuelle Untersuchungsergebnisse (z.B. persönliche Risikokonstellationen) zu erhalten.

Ich hatte ausreichend Zeit mir zu überlegen, ob ich an der Datenerhebung teilnehmen will sowie Gelegenheit Fragen zu stellen. Mit den erhaltenen Antworten bin ich zufrieden. Ich habe darüber hinaus eine Probandeninformation und eine Kopie dieser Einwilligungserklärung (datiert und unterschrieben) erhalten. Ich wurde darauf hingewiesen, dass ich jederzeit von dieser Untersuchung zurücktreten kann, ohne dass mir dadurch ein Nachteil entsteht. Die Daten werden in diesem Falle vernichtet. Ich kann auch nach der Teilnahme an dieser Studie die Löschung der hier erhobenen Daten verlangen. Ein Jahr nach Abschluss der Studie wird aber der Codierungsschlüssel gelöscht und damit ist die Zuordnung meines Namens zu meinen hier erhobenen Daten (und damit auch die Löschung der Daten) nicht mehr möglich.

Name des Teilnehmers: ..... (bitte Blockbuchstaben)

....

Ort, Datum Unterschrift des Teilnehmers

Name des aufklärenden Mitarbeiters: .....

....

Ort, Datum Unterschrift des aufklärenden Mitarbeiters

aa. Study 4: Instructions of the rating scales

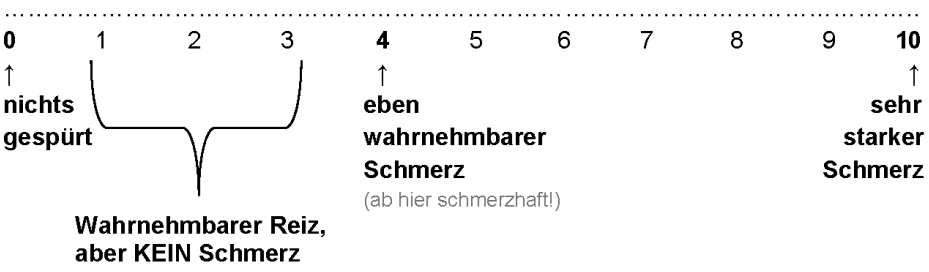
Lehrstuhl für Psychologie I - Prof. Dr. Paul Pauli
Biologische Psychologie, Klinische Psychologie und Psychotherapie

Instruktion zur Studie

Zuerst wird Ihre persönliche Schmerzschwelle bestimmt.
Ihre Aufgabe ist es, dem Versuchsleiter nach jedem elektrischen Reiz mitzuteilen, wie stark dieser war.

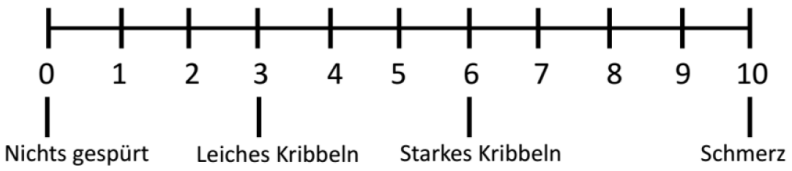
Dazu wird Ihnen folgende Frage gestellt:

Wie stark war dieser elektrische Reiz auf der Skala von 0 bis 10?



Für die Bestimmung Ihrer persönlichen optimalen Vagusnervstimulstions-Intensität werde Sie gebeten, die Stimulation selbst einzustellen. Dabei ist Ihnen der Versuchsleiter behilflich. Ihre Aufgabe:

Stellen Sie bitte die Stimulationsintensität des Vagusnervstimulators so ein, dass sie dem Richtwert „7“ auf der Skala entspricht.



Nach aktiver Erkundung der virtuellen Welt, werden sie anschließend mehrmals passiv durch die verschiedenen Räume geführt. Danach werden Ihnen Ausschnitte aus den zuvor gesehenen Räumen präsentiert und einige Fragen zu Ihren Empfindungen darin gestellt, die Sie mit Hilfe der folgenden Skalen beantworten sollen.

Wie positiv oder negativ empfanden Sie diesen Raum?

Nennen Sie dann bitte eine Zahl von **0 (sehr negativ)** bis **100 (sehr positiv)** auf der unten angegebenen Skala.

.....

0 50 **100**

Wie stark war Ihre Aufregung?

Nennen Sie dann bitte eine Zahl von **0 (gar keine Aufregung)** bis **100 (sehr starke Aufregung)** auf der unten angegebenen Skala.

.....

0 50 **100**

Wie groß war Ihre Angst?

Nennen Sie dann bitte eine Zahl von **0 (keine Angst)** bis **100 (sehr starke Angst)** auf der unten angegebenen Skala.

.....

0 50 **100**

Wie sehr haben Sie den elektrischen Reiz in diesem Raum erwartet?

Nennen Sie dann bitte eine Zahl von **0 (gar nicht)** bis **100 (ganz sicher)** auf der unten angegebenen Skala.

.....

0 50 **100**

Bitte prägen Sie sich alle Skalen gut ein. Wenn Sie später danach gefragt werden, antworten Sie bitte mündlich und möglichst spontan und zügig.
Falls Sie noch weitere Fragen haben, stellen Sie diese bitte jetzt.

bb. Study 4: Determination of ear stimulation

Schwellenbestimmung Vagusnervstimulation (1) – HAB/ ACQ

Datum: _____ VP-Code: _____

1. Stimulationsintensität: _____

2. Stimulationsintensität: _____

Mittelwert (Schwelle) : _____

Rating (Schwelle): _____

Schwellenbestimmung Vagusnervstimulation (2) – Dauerstimulation/ EXT

Datum: _____ VP-Code: _____

1. Stimulationsintensität: _____

2. Stimulationsintensität: _____

Mittelwert (Schwelle) : _____

Rating (Schwelle): _____

Schwellenbestimmung Vagusnervstimulation (3) – Test

Datum: _____ VP-Code: _____

1. Stimulationsintensität: _____

2. Stimulationsintensität: _____

Mittelwert (Schwelle) : _____

Rating (Schwelle): _____

Publication list

Publications in peer-reviewed journals

Andreatta, M., Neueder, D., **Genheimer, H.**, Schiele, M. A., Deckert, J., Domschke, K., Reif, A., Wieser, M. J., Pauli, P. (submitted). Generalization of conditioned contextual anxiety and the modulatory effects of anxiety sensitivity.

Genheimer, H., Andreatta, M., Pauli, P. (submitted). Conjunctive and elemental representation of a context in humans.

Andreatta, M., **Genheimer, H**., Neueder, D., Wieser, M. J., Pauli, P. (submitted). Context as a mediator for generalization and inhibition of conditioned fear responses.

Andreatta, M., Neueder, D., **Genheimer, H**., Schiele, M. A., Schartner, C., Deckert, J., Domschke, K., Reif, A., Wieser, M. J., Pauli, P. (2018). Human *BDNF* rs6265 polymorphisms as mediator for the generalization of contextual anxiety. *Journal of Neuroscience Research,* 97(3), 300-312.

Ventura-Bort, C., Wirkner, J., **Genheimer, H.**, Wendt, J., Hamm, A. O., Weymar, M. (2018). Effects of transcutaneous vagus nerve stimulation (tVNS) on the P300 and alpha-amylase level: A pilot study. *Frontiers in Human Neuroscience*, 12, 202.

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Published poster abstracts

Genheimer, H., Andreatta, M., Pauli, P. (2019). The more the better? Salivary alpha amylase as manipulation check for successful transcutaneous vagus nerve stimulation in context conditioning. *European Meeting on Human Fear Conditioning (EMHFC)*, Würzburg, Germany.

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Weymar, M., Ventura-Bort, C., **Genheimer, H.,** Wirkner, J., Wendt, J., Hamm, A. O. (2017). The P300 and the LC-NE system: New insights from transcutaneous vagus nerve stimulation (tVNS). *Society for Psychophysiological Research (SPR)*, Vienna, Austria.

Genheimer, H., Andreatta, M., Pauli, P. (2017). Fear generalization: New approaches of vagus nerve stimulation on fear relapse. *World Association for Stress Related and Anxiety Disorders (WASAD)*, Würzburg, Germany.

Genheimer, H., Andreatta, M., Pauli, P. (2017). The effects of vagus nerve stimulation on generalization of fear and anxiety. *European Meeting on Human Fear Conditioning (EMHFC)*, Hamburg, Germany.

Genheimer, H., Andreatta, M., Freundorfer, K., Pauli, P. (2016). Evidence for cued and contextual fear conditioning in the flip-book paradigm – The dual representation of a context. *European Meeting on Human Fear Conditioning (EMHFC)*, Utrecht, Netherlands.

Genheimer, H., Andreatta, M., Asan, E., Pauli, P. (2016). Fear Extinction in Virtual Reality – The Return of Anxiety after Extinction. *Cognitive Neuroscience Society (CNS),* New York, USA.

Genheimer, H., Andreatta, M., Asan, E., Pauli, P. (2015). Contextual Fear Conditioning – The influence of vagus nerve stimulation on fear extinction. *Psychologie und Gehirn,* Frankfurt, Germany.

Genheimer, H., Andreatta, M., Asan, E., Pauli, P. (2015). Vagus Nerve Stimulation and Fear Extinction – First Approaches towards a new therapy? *European Meeting on Human Fear Conditioning (EMHFC)*, Bochum, Germany.

Genheimer, H., Andreatta, M., Nückel K., Glotzbach-Schoon, E., Mühlberger, A., Pauli, P. (2015). Fear conditioning in a flip-book – The dual representation of cued and contextual fear. *Cognitive Neuroscience Society (CNS),* San Francisco, USA:

Genheimer, H., Spitz, L., Baumgarten, J., Gerdes, A. B. M., Mühlberger, A., Wieser, M. J. (2014). Emotional modulation of pain processing in dental phobia – Dissociation of somatosensory evoked potentials and subjective pain perception. *Psychologie und Gehirn,* Lübeck, Germany.

Genheimer, H., Reicherts, P., Wieser, M. J. (2013). Face or Race? – Processing same- or other-race faces influences the feeling of empathy. *Psychologie und Gehirn,* Würzburg, Germany.

Published talk abstracts

Genheimer, H., Andreatta, M., Pauli, P. (2019). The effects of transcutaneous vagus nerve stimulation on context dependent cue conditioning - do the contexts or the cues matter? *Society for Psychophysiological Research (SPR)*, Washington, USA.

Genheimer, H., Andreatta, M., Asan, E., Pauli, P. (2016). Transcutaneous vagus nerve stimulation and extinction learning after contextual fear conditioning in virtual reality. *Society for Psychophysiological Research (SPR)*, Minneapolis, USA.

Curriculum Vitae

Affidavit

I hereby confirm that my thesis entitled

The acquisition of anxiety and the impact of transcutaneous vagus nerve stimulation on extinction learning in virtual contexts

is the result of my own work.

I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis. Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

_____ _____

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation

Angstakquisition und der Einfluss transkutaner Vagusnervstimulation auf Extinktionslernen in virtuellen Kontexten

eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben. Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

_____ _____

Ort, Datum Unterschrift